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EXPERIMENTS ON
CORNEAL REFLEX DEVELOPMENT
IN AMPHIBIA

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOÖLOGY
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EXPERIMENTAL STUDIES ON THE DEVELOPMENT OF THE CORNEAL REFLEX IN AMPHIBIA

I. THE ONSET OF THE REFLEX AND ITS RELATIONSHIP TO METAMORPHOSIS

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FIVE FIGURES

Studies of the development of behavior in amphibians go back largely to Coghill,² who traced the development of motility in *Amblystoma* from pre-motile stages to those in which local reflexes of legs and gills were individuated from a total pattern of reaction. This work has led in recent years to an expanding study of the development of behavior in other forms (see Windle, '40). Most of these studies have been concerned with embryo and fetus, while only sporadic attention has been given to postembryonic changes in behavior.

The metamorphosis of amphibians from the larval to the adult state furnishes excellent material for studies of the development of behavior; the animals are large enough to be manipulated and observed conveniently, and their neural pathways are essentially complete. The behavioral changes at metamorphosis are as striking as the morphological changes which they accompany. In the anurans these behavioral

¹ This research was done under the direction of Dr. Paul Weiss in partial fulfillment of the requirements for the degree of Doctor of Philosophy. It has been aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of The University of Chicago. A preliminary report appeared in *The Anatomical Record* (Kollros, '41).

² See Coghill ('29) for a review of his earlier work. For a detailed review of the literature, see Hooker ('36).

changes include the establishment of the use of the hind limbs in locomotion and postural adjustment, and modifications in the use of the jaws and tongue associated with a change in food habits from a browsing omnivorous type to a predacious carnivorous one. Urodeles need not make these adjustments at metamorphosis, for they develop limbs early in larval life and are exclusively carnivorous. Common to both groups is the transition from aquatic to aerial respiration, and the first appearance of molting. Another change found in both groups, the one reported on in this paper, is the development of the corneal reflex. In the adult this reflex is elicited by tactile stimulation of the cornea and consists of a retraction of the bulb into the orbit. In the larva the eye remains immobile when the cornea is stimulated.

Earlier papers mentioning the corneal reflex present conflicting claims as to the time of its onset. In large larvae of *Pelobates fuscus*, Harms ('23) noted retraction of the bulb when the cornea was stimulated. Giesbrecht ('25) observed in *Rana esculenta* that strong stimulation of the outer cornea with a blunt needle evokes the retraction of the eyeball in tadpoles whose hind limbs have not yet begun to function, and that with the approach of metamorphosis the strength of the stimulus required to evoke the reflex diminishes. It was reported by Koppányi ('23) that the corneal reflex can be evoked in old (exact age not stated) larvae of *Salamandra maculosa*. In contrast with these views Petersen ('24) stated that the corneal reflex does not appear premetamorphically in either urodeles or anurans. L. S. Stone ('30) reported that the reflex is first obtainable in *Amblystoma* at metamorphic climax, and later ('38) he reaffirmed that the corneal reflex cannot be obtained until the adult stage. The present paper is devoted to a description of the appearance of the reflex and to experiments dealing with the relationship of the time of onset of the reflex to metamorphosis. A later paper will deal with experiments which attempt to locate the factors responsible for the development and onset of the reflex.

MATERIAL AND METHODS

The animals used for this study were larvae of *Amblystoma tigrinum*, *A. punctatum*, *A. opacum*, the axolotl, *Triturus torosus*, *T. viridescens*, *Rana catesbiana*, *R. pipiens* (collected in nature or obtained by pituitary treatment), *Hyla crucifer*, *H. versicolor versicolor*, *Pseudacris nigrita triseriata*, and *Bufo fowleri*. The adults of all of these were also used, and in addition, adults of *Necturus*, *Amblystoma jeffersonianum*, *Triturus pyrrhogaster* and *Plethodon cinereus*. Larvae were kept in individual fingerbowls, anurans being fed on boiled lettuce, and urodeles on *Daphnia*, mosquito larvae and pupae, *Enchytraeus*, and liver.

A human hair mounted in a capillary glass tube from which it protruded from 5 to 7 mm. was used for the application of tactile stimuli. The hair was always applied with its side rather than its tip. Stimulation will be designated as "light", "medium", and "strong". Light stimulation was produced by bending the hair but slightly upon touching the animal; medium stimulation by bending it from 15° to 30°; strong stimulation by bending it more than 30°. All observations and stimulations of larvae were made under the low power (20 X) of a wide-field binocular dissecting microscope. Stimulation of the larvae was done under water, and of adults in air. Except for some overexcitable specimens of *Amblystoma tigrinum*, and those bloated with air and floating with their ventral surface upward, all animals were permitted to move freely during the tests.

ANATOMICAL BASIS OF THE REFLEX

The corneal reflex of the adult amphibian is elicited by tactile stimuli applied to the cornea or to a circumscribed area about it. The reflex consists of the withdrawal of the bulb into the orbit through the action of the retractor bulbi muscle. The tendon of the nictitating membrane lies under the retractor bulbi muscle and passes upward to insert upon the dorsal edge of the nictitating membrane at both the rostral and caudal

borders of the eye. Contraction of the retractor bulbi muscle stretches this tendon and pulls the membrane up. This results in a passive closure of the lids. The eye is returned to its original position by the action of the levator bulbi muscle. The retractor bulbi muscle is innervated by the abducens nerve (Ecker, Wiedersheim und Gaupp, 1896; Francis, '34). The reflex path includes the cornea, trigeminal nerve, Gasserian ganglion, centers in the brain, abducens nerve, and the retractor bulbi muscle.

A. Urodeles

Sensory apparatus. Sections of *Amblystoma* embryos stained according to Bodian's method ('36, '37) reveal that the cornea is supplied with nerve fibers from the trigeminal ganglion as early as in the pre-feeding stage.

Motor apparatus. According to Piatt ('38) the anlage of the retractor bulbi muscle is present in *Amblystoma punctatum* at Harrison's stage 46, while the anlage of the levator bulbi muscle is not recognizable until some time later, when the larvae have reached a length of 18 or 19 mm. The retractor bulbi muscle has its origin partly on the parasphenoid bone, but also partly on the orbitosphenoid (Francis, '34). It inserts upon the bulb just ventral to the optic nerve in a broad, shallow, dorsally directed arc. Much of its insertion is covered by the inferior and lateral rectus muscles. In the young animal the most anterior fibers of the muscle are nearly twice as long as the most posterior ones, but with increasing age the muscle broadens and its origin shifts toward the midline of the skull; as a result, its fibers are more nearly aligned with the medio-lateral axis of the eyeball than they are in the younger animal. Whether this shift of the origin toward the midline is due to changes in the muscle, in the skull, or in both, was not determined.

In the urodele larvae the cornea is at all times connected to the conjunctiva, and the bulb is unable to move with the freedom displayed by the bulb of the young anurans.

B. Anurans

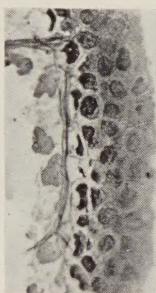
Sensory apparatus. Silver impregnations of sections of the anuran cornea reveal that it contains nerve fibers as early as in the pre-feeding stage (fig. 1).

Motor apparatus. Dissections of *Rana catesbiana* and *R. pipiens* show that the retractor bulbi is present as a thin, narrow band in tadpoles whose hind limbs are in the bud stage. Sections of the muscle at this time reveal that the motor nerve fibers are present and that the muscle fibers are developing cross striations (fig. 2). The muscle originates on the para-

Fig. 1

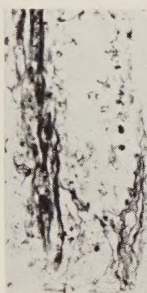


a



b

Fig. 2



a



b

Fig. 1 Photomicrographs of (a) tangential and (b) radial sections through the cornea of a 45 mm. *Rana pipiens* tadpole, showing sensory nerve fibers. $\times 230$.

Fig. 2 Photomicrographs of sections of the retractor bulbi muscle in (a) 37 mm. and (b) 42 mm. (1 week older) *Rana pipiens* tadpoles. Note in (a) that the bundle of nerve fibers is separated from the developing muscle by a loose mesenchyme, traversed by only a few nerve fibers. Note well developed cross striations in (b).

sphenoid bone (Ecker, Wiedersheim und Gaupp, 1896). As development proceeds, the size of the muscle increases slowly until metamorphic climax, when its growth and that of the other extrinsic eye muscles becomes very rapid. This increase in muscle size has been reported for *Bufo* by Romano ('36). Throughout this period of growth, and particularly at metamorphic climax, the origin of the muscle shifts mesiad from its original lateral position, until it eventually lies almost

in the midline of the skull (fig. 3). The direction of its fibers then coincides with the medio-lateral axis of the eyeball, whereas in the young tadpole the fibers are directed more posteriorly. The insertion of the retractor bulbi muscle upon the bulb is essentially as in urodeles, in a broad, shallow arc just below the optic nerve.

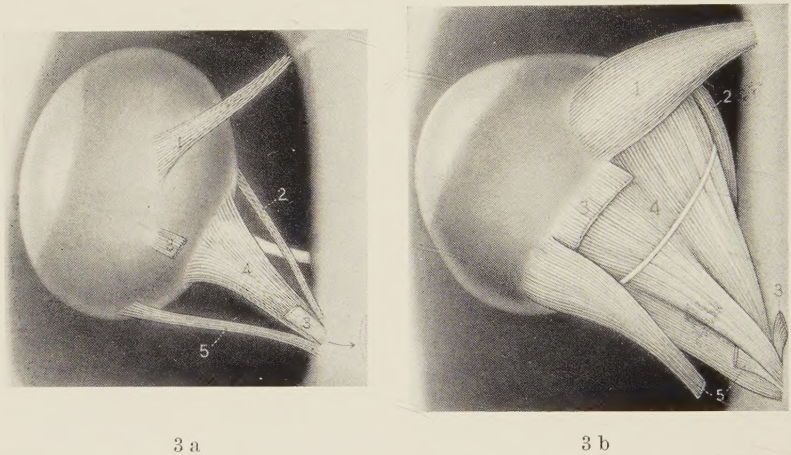


Fig. 3 a, Right eyeball of 45 mm. *Rana pipiens* tadpole. Ventral view. $\times 13$. b, Right eyeball of adult *Rana pipiens*. $\times 5$. 1, Musculus obliquus inferior. 2, M. rectus medialis. 3, M. rectus inferior. 4, M. retractor bulbi. 5, M. rectus lateralis. In (a) the optic nerve is seen dorsal to the retractor bulbi and medial rectus. The arrow indicates the future migration of the origin of the muscles toward the midline (right edge of picture). In (b) the white band passing over the surface of the retractor bulbi muscle is the tendon of the nictitating membrane.

The cornea of the tadpole differs from that of the urodele larva in that it is composed of two layers, separated by an intra-orbital fluid. This permits the bulb to move freely. The so-called cornea of the larval eye is formed from the external conjunctiva and the epidermal cornea, and is separated from the internal cornea, or membrana descemetii, which is continuous with the sclera. At metamorphic climax these two corneae fuse (Harms, '23; Petersen, '24).

METAMORPHOSIS

A. Urodeles

Changes in the pigmentation of the larvae appear 2 to 3 weeks before the reduction of the external gills begins. Skin glands develop. The anterior extension of the dorsal fin upon the neck region starts to recede. It is during this preparatory phase that the corneal reflex develops and the eyelids begin to form. With the onset of metamorphic climax the animal stops feeding; its gills and tail fins are resorbed; the skin thickens, becomes cornified, and begins to be shed; the eyes protrude and the lids grow; the contour of the head changes; the limbs become stouter; the skin darkens, and a few days later (*Amblystoma punctatum*) the yellow spots characteristic of the adult make their first appearance; feeding is resumed.

B. Anurans

In anurans, similarly, metamorphic climax is preceded by a period of premetamorphic changes, characterized by the rapid growth of the hind limbs externally and of the forelimbs within the branchial cavity. During this period glands have appeared in the skin, and the adult pigment pattern has been gradually developed. At metamorphic climax feeding stops; the forelimbs emerge through holes in the operculum; the beak is lost, and the mouth is formed; the entire tail is resorbed; the gills are resorbed and the operculum closes; a tympanum is formed; feeding is resumed.

Throughout this paper the onset of metamorphosis will be taken to mean the start of metamorphic climax, marked in the urodeles by the beginning of gill reduction, and in the anurans by the emergence of the forelimbs.

NORMAL DEVELOPMENT OF THE CORNEAL REFLEX

A. Urodeles

1. *Amblystoma tigrinum*. In *Amblystoma tigrinum*, under conditions of feeding approaching maximal, the reflex appears

when the larva has reached a length of 65 to 112 mm. Metamorphic climax occurs at 86 to 114 mm. (table 1). Some animals display the reflex for as much as 3 weeks before metamorphosis, while others manifest it for only 2 or 3 days before the onset of that event. The anterior half of the cornea and the skin bordering the rostral edge of the eye are the regions from which the reflex can be first evoked. The reflexogenous area expands gradually to include successively the entire cornea, the lids, and finally a region of the head which may extend rostrad to the nostril, dorsad to the midline, and caudad to one-third, or in extreme cases, almost one-half of the distance from the eye to the base of the gills. The reflexogenous area keeps expanding throughout the larval period; at metamorphic climax, however, the maximum extent has been reached, and this area is maintained by the adult (fig. 4). Animals which develop the reflex late in larval life invariably have a smaller reflexogenous area at metamorphosis than do those animals which display the reflex earlier in development. In the latter group the retraction of the bulb is at first associated with forward rotation, while in the former group it appears from the start as straight retraction without a rotational component. Apparently the development of the retractor bulbi muscle proceeds at about the same rate in the two groups; however, in the animals which display the reflex early, the origin of the retractor bulbi has not yet reached its final position and, being excentric, causes a slight rotation. Larvae which have displayed the reflex for some time are found to respond to stimuli of lesser strength, and this decline in the threshold continues concurrently with the spread of the reflexogenous zone. With increasing distance from the cornea the strength of the stimulus required to evoke the reflex rises rapidly.

The corneal reflex in both larvae and adults exhibits spatial and temporal summation. When the reflex is first displayed it can be elicited only by stroking over a large portion of the sensitive region of the cornea, whereas stimulation of single points is ineffective; but if stimuli to single points are rapidly

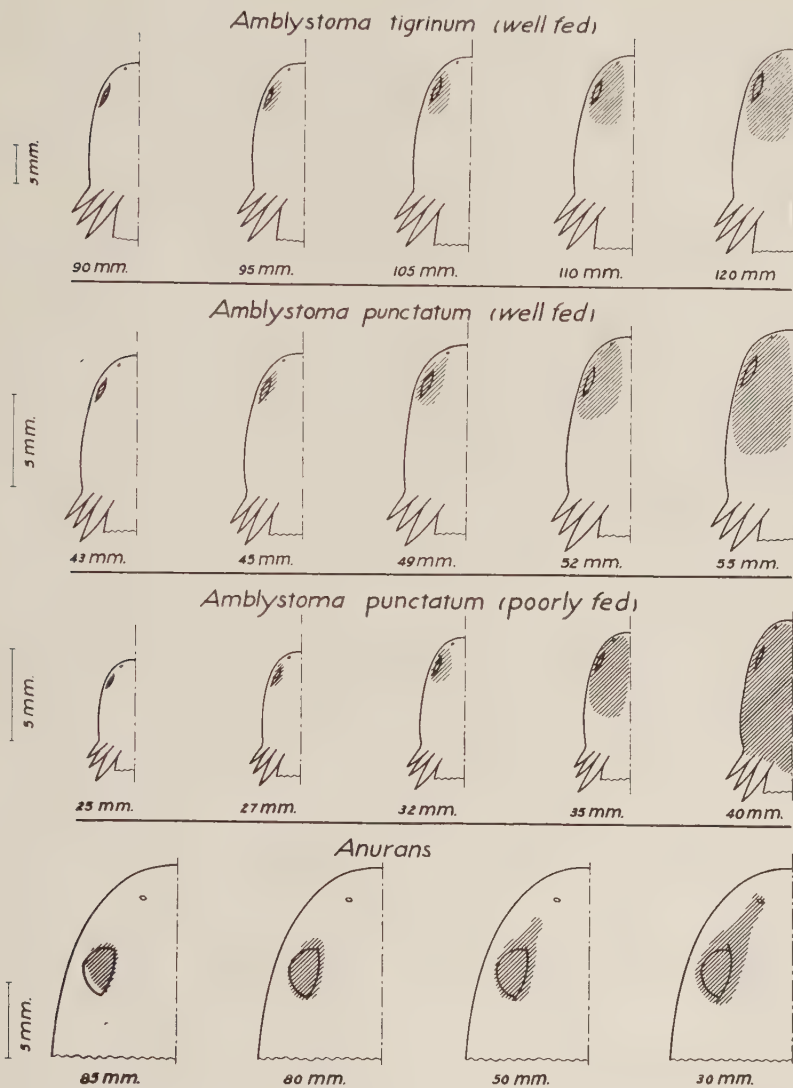


Fig. 4 Expansion of the reflexogenous area in well fed *Amblystoma tigrinum*, well fed *A. punctatum*, poorly fed *A. punctatum*, and in anurans. Typical total lengths of the larvae are indicated at the various stages. For anurans the length of metamorphosing *Rana pipiens* was used. The last figure for poorly fed *A. punctatum* shows median overlap of reflexogenous areas at the level of the eyes.

repeated, the reflex may be elicited. Summation is best demonstrated in the corneal region just after the onset of the reflex, and in the extra-corneal region some time later.

The typical response of the gills to tactile stimuli applied to the head is depression. At first, before the corneal reflex is elicitable, weak stimuli applied to the cornea evoke only a depression of the ipsilateral gills; later this response is accompanied by retraction of the bulb; while still later the retraction of the bulb can be obtained without a depression of the gills if a very weak stimulus is employed. Strong stimuli, even if applied to but a single point, may augment the response of the gills from an ipsilateral to a bilateral depression, but it requires an even stronger stimulus to call forth a retraction of both eyeballs.

2. *Amblystoma punctatum* and *Amblystoma opacum*. *Amblystoma punctatum* and *A. opacum* are similar in regard to the time of development of the corneal reflex. If approximately maximal feeding has been maintained, the reflex can be obtained first when the larvae measure from 39 to 52 mm. in length. Metamorphosis occurs at 46 to 64 mm. All animals display the reflex at least 1 week before metamorphosis, and some may display it 3 weeks or more before that time (table 1). When the reflex is first elicitable, it has a rotational component, but this persists for only a few days. The reflex can be first evoked from the cornea and from the skin just rostrad to it, but the reflexogenous area spreads rapidly rostrad, dorsad, and caudad, extending to about three-fifths of the distance from the eye to the base of the gills before metamorphosis occurs (fig. 4). As in *Amblystoma tigrinum*, the extent of the reflexogenous area of the metamorphosed *A. punctatum* corresponds to its extent just before metamorphosis.

In *A. punctatum* and *A. opacum* the reaction of the gills to stimulation of the cornea and the adjacent region is essentially as in *A. tigrinum*, except that just before metamorphosis the threshold for the corneal reflex is distinctly below that

for gill depression. In old, just premetamorphic larvae of *Amblystoma punctatum* the following definite sequence of reactions to corneal stimulation of increasing strength can be observed:

1. Moderate retraction of the ipsilateral eye.
2. Stronger retraction of the ipsilateral eye and depression of the ipsilateral gills, particularly the third gill.
3. As in (2), with strong depression of all three ipsilateral gills.

TABLE 1

Total length of well fed Amblystoma larvae at the onset of the corneal reflex and at metamorphic climax.

AMBLYSTOMA TIGRINUM				AMBLYSTOMA PUNCTATUM			
Onset of reflex		Metamorphosis		Onset of reflex		Metamorphosis	
<i>Length in mm.</i>	<i>No. of animals</i>	<i>Length in mm.</i>	<i>No. of animals</i>	<i>Length in mm.</i>	<i>No. of animals</i>	<i>Length in mm.</i>	<i>No. of animals</i>
65-70	2			39-40	7	46-47	1
71-75	1			41-42	3	48-49	4
76-80	6			43-44	2	50-51	4
81-85	5			45-46	2	52-53	7
86-90	8	86-90	5	47-48	3	54-55	13
91-95	7	91-95	4	49-50	4	56-57	5
96-100	3	96-100	11	51-52	1	58-60	2
101-105	0	101-105	6			61-64	1
106-110	0	106-110	2				
111-115	1	111-115	5				
Ave. 92.3	33	Ave. 99.8	33	Ave. 44.3	22	Ave. 53.5	37

4. Strong retraction of the ipsilateral eye, and bilateral depression of the gills.
5. As in (4), with bending of the head away from the stimulus.
6. As in (5), with adduction of the ipsilateral upper arm.
7. As in (5), with retreat from the stimulus.
8. Precipitate swimming away.

Steps 1, 3, and 8 can be obtained in all animals, steps 2, 4, 5, and 7 in many, and 6 in only the least excitable and the oldest ones. For any given animal, from day to day, the same sequence of responses was noted. Infrequently, however, devi-

ations from the pattern of response occurred; generally these were due to an increased excitability of the animal, which would swim away precipitately before the test had proceeded to the later steps.

Variability in response was not found with respect to the extent of the reflexogenous area, except as that area increased. If a response was once obtained from a given region, it could be obtained from that same region upon any subsequent occasion. The full array of responses is rarely demonstrable in *A. tigrinum* larvae, owing to their greater excitability. In these, as in some *A. punctatum* larvae, step 8 supersedes all other grades of response.

3. *Axolotl* (*Amblystoma mexicanum*). Only four axolotl larvae were studied. In these the reflex appeared when they had reached lengths of 55 to 63 mm. As in *A. tigrinum*, the reflex was first restricted to the cornea, but soon the area from which it could be evoked increased in extent. When the larvae had grown to about 110 mm., the reflex could be elicited from the entire dorsal surface of the head, although near the base of the gills the threshold was very high.

4. *Triturus*. Maximally fed larvae of *Triturus torosus* first display the corneal reflex when they are 30 to 39 mm. long; they metamorphose at lengths of 37 to 49 mm. Except for the greater spread of the reflexogenous area in *Triturus*, in which it includes the entire dorsal surface of the head, the reflex develops as in *Amblystoma punctatum*.

In a single specimen of *Triturus viridescens* the reflex developed at 37 mm., and metamorphosis occurred at 55 mm. The reflexogenous area was of the same extent as in *Amblystoma punctatum*.

5. *Other forms*. Single adult specimens of *Necturus* (33 cm.), *Amblystoma jeffersonianum*, and *Plethodon cinereus* were examined. *Necturus* has no corneal reflex, although slight pressure upon the eye depresses it, indicating that the orbit is sufficiently large to permit bulbar retraction. It is of interest to note that *Necturus* is lidless, whereas the mature

axolotl, which shows the reflex, possesses lids. The reflexogenous area of the specimen of *Amblystoma jeffersonianum* included the entire dorsal surface of the head. That of *Plethodon cinereus* was as great in extent as that of the adult *Amblystoma tigrinum*. Several adult specimens of *Triturus viridescens* and *T. pyrrhogaster* were also tested; in both species the reflex was elicitable by ordinary stimuli only from the cornea and the lids. Much stronger stimuli (blunt teasing needle) were required to elicit the reflex from more posterior parts of the head. In this restriction of the reflexogenous area, these adults differ from recently metamorphosed *Triturus torosus*.³

B. Anurans

1. *Rana pipiens*. In well fed *Rana pipiens*, about 12 days before the emergence of the forelimbs, the dorsal skin spots characteristic of the adult first appear. Originally they form circles on the tadpole's back, being best seen from a lateral view. They darken slowly, and it is not until some 4 days later that they stand out clearly against the lighter background. After another 3 or 4 days the corneal reflex appears. This usually antedates the emergence of the forelimbs by 3 or 4 days. In rare cases the reflex precedes forelimb emergence by as much as 7 to 10 days. Invariably the reflex can at first be elicited only by very strong stimuli; later weaker ones suffice. The regions from which the reflex can be elicited are first the anterior half of the cornea, later the entire cornea, and still later the lids and the skin rostrad of the eye to the nostril (fig. 4). In more than forty cases, without exception, the reflex was more readily elicited by stroking the cornea than by merely touching it with equal pressure. This demonstrates spatial summation. Later, when the reflex is established, light touch alone is a sufficient stimulus on the cornea,

³ Dr. Paul Weiss has informed me in a personal communication that in *Triturus torosus* the reflexogenous area shrinks after metamorphosis; after 3 months the area rarely extends as far back as the level of the inner ear. Thus the adults of this species fall in line with *T. viridescens* and *T. pyrrhogaster* with respect to the restriction of the reflexogenous area.

but at the periphery of the reflexogenous area (skin beyond the lids) the reflex usually cannot be evoked except by rather forceful stroking.

2. *Rana catesbiana*. In *Rana catesbiana* the reflex appears much as in *R. pipiens*, several days before the emergence of the forelimbs. In two cases out of twenty-two, however, it appeared prematurely, by at least 1 month in one individual, and 6 to 8 weeks in the other, which died before metamorphosing.

3. *Hylidae*. In the three species of *Hylidae* studied, *Hyla versicolor versicolor*, *H. crucifer*, and *Pseudacris nigrita triseriata*, the reflex appears during the period of tail resorption, after the forelimbs have emerged. When the forelimbs emerge, the tail measures from two to two and one-half times the length of the body, with the total length ranging from 28 to 45 mm. With but two exceptions in the sixty-three animals studied, the corneal reflex first appeared in the 24-hour period during which the tail shrank from one and one-half times the length of the body to one-third of the body length.

In both *Rana* and the *Hylidae* the *membrana descemetii* had fused to the external cornea before the reflex appeared, except in the two *Rana catesbiana* tadpoles which developed the reflex prematurely.

4. *Bufo fowleri*. The tadpoles of *Bufo fowleri* have a black back, which under the dissecting microscope is seen to be mottled with small yellow rods and dots. Shortly after the hind limbs enter their period of rapid growth, certain areas of the back lose the yellow pigment, apparently by migration. A day or two later the yellow dots reappear diffusely throughout the black areas and become rapidly concentrated into two larger, flat yellow spots in each of these areas. This period of reinvasion and aggregation requires approximately 30 to 48 hours, and is succeeded by a period in which the large yellow spots become raised into warts. It was almost invariably during the first day of wart formation that the corneal reflex appeared; in a few of the sixty animals observed it occurred shortly before this, and in a few others shortly after.

It always developed before the emergence of the forelegs, which occurred 3 or 4 days after the formation of the flat, yellow spots. In *Bufo* the reflex appeared before the membrana descemetii became fused to the external cornea, contrasting with the condition found in *Rana* and the *Hylidae*.

In all anurans studied the reflex is restricted to the cornea, the lids, the skin directly surrounding them, and the skin between the nostrils and the eye.

EXPERIMENTAL

The development of the corneal reflex in relation to metamorphosis has been set forth in the preceding section. It had previously been noted that although the innervation of the cornea and the condition of the retractor bulbi muscle in mid-larval stages appear anatomically adequate for the corneal reflex, nevertheless, the reflex normally does not appear until shortly before metamorphosis, or at best, in maximally fed animals, a few weeks before metamorphosis. The ability of the peripheral components (sensory and motor) of the reflex to function in the midlarval stages can be readily demonstrated.

Functional condition of the corneal innervation

If the cornea of the young salamander larva is touched lightly, the animal responds by swimming away; older larvae respond to light touch on the cornea by a depression of the gills of the same side or of both sides, swimming away only if the stimulus is increased. The anuran tadpoles reveal corneal sensibility by swimming away when the cornea is touched.

Functional capacity of the motor components

The retraction of the eyeball cannot be elicited by stimulation of the cornea in half-grown larvae of either of the amphibian orders. It can be obtained, however, by applying induction shocks to any part of the motor path of the future reflex. The test animals included a graduated series of eight

Rana catesbiana tadpoles (45 to 84 mm.), four *R. pipiens* tadpoles (40 to 65 mm.), and five half-size (50 to 60 mm.) *Amblystoma tigrinum* larvae. In none of these animals could the reflex be elicited by stroking externally.

In preparation for electrical stimulation the animals were anesthetized in chloretone. Their heads were removed and the lower jaws were discarded. Eyebulbs, eye muscles and their nerves, and the medulla oblongata were exposed from the ventral side. The head was then placed in amphibian Ringer's solution for 20 minutes to permit recovery from the anesthetic. The testing was carried out in the Ringer's solution, shocks being applied from the secondary of a Harvard induction coil. Faradic stimulation was used.

A. Urodeles. The electrodes were first applied to the retractor bulbi muscle. A contraction of the muscle resulted. Then, after adjusting the induction coil so as to deliver shocks of slightly supra-liminal intensity, the electrodes were applied to the other eye muscles; these contracted in turn, but the retractor bulbi did not. All of the extrinsic eye muscles except the retractor bulbi were then cut. Any subsequent movement of the bulb could thus be ascribed to the contraction of the retractor bulbi. Stimulation of the abducens nerve and of the medulla in the region of the abducens center led to a contraction of the retractor bulbi muscle, with rotation and retraction of the bulb. The electrodes were then placed in positions posterior and anterior to the abducens center, and finally in various sites in the vicinity of the eye. At none of these sites did stimulation produce contraction of the muscle. Obviously, the previous stimulation of the abducens nerve and abducens center must have been transmitted to the muscle by way of the nerve, rather than by way of "escape" currents in the Ringer's solution. This is further confirmed by the fact that upon returning the electrodes to the abducens center after the abducens nerve had been sectioned, the retractor bulbi muscle did not contract.

B. Anurans. The experiments detailed above for the urodeles were repeated on *Rana*, and analogous results were obtained in all except the three smallest (45 mm.) *R. catesbiana* tadpoles. In these latter the retractor bulbi responded with a slow contraction rather than with a quick twitch, probably an expression of the embryonic condition of the muscle at that stage.

*Relationship of the onset of the corneal reflex
to metamorphosis*

In the previous section the ability of the peripheral components of the corneal reflex to function in the midlarval stage has been demonstrated. As it was shown above, however, the reflex fails to appear in maximally fed animals until shortly before metamorphosis. This suggested some definite relationship between the onset of the reflex and other metamorphic events, and possibly even a common causation. To test this possibility experiments were carried out in which metamorphosis was delayed, wholly suppressed, or advanced.

*I. Delay of metamorphosis by maintenance of low
nutritional levels*

A. Urodeles. The growth of *Amblystoma tigrinum* and *A. punctatum* under conditions of maximal feeding has been described by Twitty and Schwind ('31). Submaximal feeding decreases the growth rate, and if feeding is sufficiently restricted, growth may be stopped completely for certain periods, according to Twitty and Elliott ('34). Concomitant with the inhibition of growth, there is a delay in the onset of metamorphosis. *Amblystoma punctatum* larvae begin feeding at about Harrison's stage 46. At this stage they are about 17 mm. in length. If they are subsequently fed maximally they will grow to 20 mm. in about 6 days, and for the following month they will grow approximately 1 mm. per day. Finally, when they are 50 to 55 mm. in length (40 to 45 days after the start of feeding), they metamorphose. Generally there is a

decline in the growth rate in the last few days preceding metamorphosis. *Amblystoma tigrinum* grows at about twice the rate of *A. punctatum*, that is, at about 2 mm. per day, until reaching lengths of 100 to 120 mm., and then it metamorphoses, at about 60 days after the start of feeding. The values just given for growth rate, larval period, and larval size vary according to the local races of *Amblystoma* used.

1. *Amblystoma punctatum*. The appearance of the corneal reflex in *Amblystoma punctatum* larvae which had been fed nearly maximally has already been described. If feeding is radically reduced, almost to a maintenance level, growth becomes extremely slow. The larval period is greatly prolonged, frequently to 10 months or more, which is more than four times the normal larval span. Under such conditions the corneal reflex can be demonstrated when the larvae are but 25 mm. long, and all larvae exhibit the reflex before reaching a length of 30 mm. In these animals the reflex may have a rotational component alone for more than a month, but it may also display only retraction for as much as 3 months before metamorphic climax. It can, therefore, be inferred that the shifts in the muscle origin and insertion which normally take place just before and during metamorphic climax have occurred prematurely in the poorly fed animals. Apparently metamorphic climax is more inhibited than are the premetamorphic changes which characterize the development of the corneal reflex. Other precocious changes noted are in the pigment pattern (development of large, lateral yellow spots), development of skin glands, partial development of the lids, and to a lesser degree, the regression of the dorsal fin upon the trunk. Metamorphic climax, when it finally takes place, appears to be quite normal. The reflex is evokable first from the cornea, then from the skin between the nostril and the eye, and from the lids. In these respects the reflex behaves exactly as in the maximally fed animals. The reflexogenous area continues to spread, and finally in most specimens covers the entire dorsal surface of the head (fig. 4). After meta-

metamorphosis the animals retain this abnormally large reflexogenous area. The response to weak stimulation is generally unilateral, but in some animals light stimulation of the median region between the eyes results in retraction of the bulbs of both sides (fig. 4). This median overlap of the lateral reflexogenous areas is seldom seen in maximally fed animals. If intermediate feeding levels are maintained, intermediate values are obtained for the time necessary for the establishment of the reflex, for the loss of its rotational component, for the spread of the reflexogenous area, and for metamorphosis; in short, for all of the features in the development of the corneal reflex, for other premetamorphic changes, and for the initiation of metamorphosis as well. As the degree of inanition is increased, the age of the animals at the onset of the reflex is also increased, as is also their age at metamorphosis. In well fed animals the period between the onset of the reflex and metamorphosis seldom exceeds 3 weeks; in poorly fed ones it may exceed 5 months. With inanition the range of the period of initiation of the reflex is extended, while the variability in the size of the animals during this period is decreased. The magnitude of the discrepancies between well fed and poorly fed animals depends upon the degree of inanition. These relationships are expressed in figure 5.

2. *Amblystoma tigrinum*. *Amblystoma tigrinum* larvae, when subjected to differential feeding, show the same responses as do *A. punctatum* larvae under the same conditions: delay of both metamorphosis and the appearance of the corneal reflex in inanition, with less delay in the development of the reflex than in general metamorphosis, and a greater spread of the reflexogenous area before metamorphosis. In animals whose larval period has been extended from a normal value of approximately 3 months to 6 or 8, the reflex can be first evoked when they are from 50 to 55 mm. long, and all of them will display the reflex before they reach a length of 60 mm. Metamorphosis also occurs at a smaller size (80 to 105 mm.) than in well fed animals (86 to 114 mm.). The

reflexogenous area also extends farther posteriorly in the older larvae, until it reaches one-half or even more of the distance from the eye to the base of the gills; in one case only did the reflexogenous area extend over the entire head. The adults retain the expanded reflexogenous area.

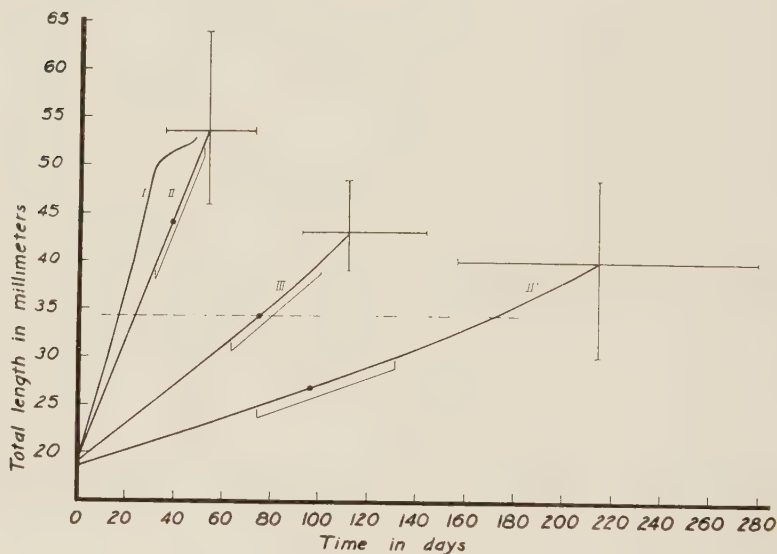


Fig. 5 Smoothed growth curves of *Amblystoma punctatum* under different conditions of feeding. Curve I expresses growth at maximal feeding, according to Twitty and Schwind ('31); curve II represents forty-eight well fed animals; curve IV represents sixteen poorly fed ones; curve III represents ten animals fed at intermediate levels. The curves terminate at the average length and age at metamorphosis. The vertical lines represent the variability in length at metamorphosis, and the horizontal lines represent the variability in age at that time. The brace on the curve represents the range in length for the onset of the reflex, and the dot indicates the average length at that time.

3. *Triturus torosus*. Poorly fed *Triturus torosus* develop the reflex when they are but 26 to 30 mm. long, in contrast to well fed animals, which develop it when they are from 30 to 39 mm. in length. The subsequent spread of the reflexogenous area is much slower than in well fed animals, and metamorphosis is considerably delayed. Thus inanition affects *Triturus*, in these respects, much as it does *Amblystoma*.

B. Anurans. Adolph ('31) demonstrated that tadpoles whose growth is inhibited are delayed in their metamorphosis. When metamorphosis finally begins, the tadpole is still considerably smaller than its uninhibited control. It has been shown by D'Angelo, Gordon, and Charipper ('41) that this delay may be due to atrophic changes in the thyroid and pituitary glands.

In the present experiments the growth of *Rana pipiens* tadpoles was retarded by restricting the amount of food available to them. In contrast to the usual larval period of 3 months, the larval period of these inhibited animals extended to 6 and even 9 months. The corneal reflex in these animals, nevertheless, appeared at the same stage as in the well fed controls, that is, 3 or 4 days before the emergence of the forelimbs. Metamorphosis proceeded normally, but the animals were smaller than their controls. The extent of the reflexogenous area was the same in poorly fed and well fed animals.

Obviously frogs and salamanders differ with respect to the effects of inanition upon the development of the corneal reflex. In the former the onset of the reflex cannot be dissociated from the other events of metamorphosis, whereas in the latter group such dissociation can be effected.

II. Suppression of metamorphosis by hypophysectomy

The metamorphosis of amphibians is dependent upon the release of hormone from the thyroid gland. The production of the thyroid hormone is in turn dependent upon another hormone elaborated by the pituitary body (Smith, '13). It is possible, therefore, to suppress metamorphosis completely by removing either the thyroid gland or the pituitary body before metamorphosis is initiated. This is appropriately accomplished by removing the oral anlage of the hypophysis in embryos of the tail bud stage. Such animals will fail to undergo metamorphosis unless the deficiency of thyrotropic hormone is artificially corrected by implants of the pituitary body, or injections of extracts of the anterior lobe of the pituitary (Smith, '22), or by the administration of thyroid extracts.

A. Urodeles. The oral anlage of the hypophysis was successfully removed from six embryos of *Amblystoma punctatum* at the tail bud stage. The reflex appeared, nevertheless, and at a time when the animals were somewhat smaller than their controls, that is, at 33 to 38 mm. as contrasted to 39 to 52 mm. This was to be expected, since the animals grew at a somewhat reduced rate, as had already been observed by Blount ('35). The reflexogenous area spreads much as in well fed animals, although it is less restricted in extent. By the time the larvae had achieved a length of 50 mm., the rotation of the eyeball had given way to pure retraction. Normal animals generally metamorphose before reaching a length of 60 mm. Of the six hypophysectomized animals, four died without metamorphosing after reaching lengths of 55 to 67 mm.; one larva lived 38 weeks and grew to a length of 70 mm., and the remaining animal is still alive in the larval condition after 80 weeks. It has a length of 99 mm. The reflex was elicitable from nearly the entire dorsal surface of the head of the 70 mm. animal, and it is now elicitable from the entire dorsal surface of the head of the surviving one. These results, in conjunction with those from differential feeding, prove that in *Amblystoma* the onset of the corneal reflex can be separated from metamorphic climax. To substantiate this conclusion further, the oral anlage of the hypophysis was removed in embryos of *Amblystoma tigrinum* too. Results obtained with these duplicate completely those in *A. punctatum*, that is, the reflex develops somewhat later than normal because of the slower growth rate, but when it is first evidenced the animals are smaller than their controls. The spread of the reflexogenous area continues beyond the normal limits, and the larvae grow considerably beyond the normal size limit at metamorphosis.

B. Anurans. The oral anlage of the hypophysis was removed in ten *Rana pipiens* embryos at the tail bud stage. The typical silvery condition of the tadpole resulted (Smith, '13, '20). Five specimens died between the ages of 6 and 9 months, in larval condition, without ever having developed the reflex. The five which are still alive after 80 weeks have likewise

failed to develop the reflex, although their larval period has been extended to more than six times its normal length. Apparently, in contrast to *Amblystoma*, the onset of the corneal reflex cannot be induced premetamorphically in *Rana* by extending the larval period.

III. Acceleration of metamorphosis by thyroxin treatment

This series of experiments is concerned only with anurans. Ten milligrams of thyroxin crystals were dissolved in one liter of water. This stock solution was added dropwise to the finger-bowls in which the animals were kept. Concentrations of thyroxin were increased gradually, since according to Etkin ('35) this method produces an essentially normal metamorphic sequence. The initial concentration of 5×10^{-9} was raised to 2 or 3×10^{-7} within periods of 2 to 6 weeks, and for four animals it was raised to 1×10^{-6} .

1. *Rana pipiens*. Ten pairs of tadpoles from 27 to 31 mm. in length were subjected to different mounting dosages of thyroxin. They metamorphosed in from 24 to 41 days, while a control group of ten similar animals required an average of 53 days. Of the treated group, the least accelerated members showed no dislocation of the reflex with respect to other metamorphic events; but those animals whose metamorphosis had been advanced by 3 weeks did evidence a slight dislocation in the time of onset of the reflex, for this occurred 2 days after the emergence of the forelimbs rather than a few days before their emergence. Those animals showing the greatest metamorphic acceleration were highly abnormal, for the forelimbs emerged when they, as well as the hind limbs, were still in the early stages of digit formation. The body form was also distorted, the anterior half being proportionately too large. These animals died 4 days after the forelimbs emerged, without having displayed the reflex. Two other tadpoles, of 55 mm., with hind limbs in an early bud stage, had their metamorphosis hastened by several weeks; they first evinced the reflex 2 days after the emergence of the forelimbs.

2. *Rana catesbiana*. Two *Rana catesbiana* tadpoles with small, non-functional hind limbs were subjected to thyroxin treatment. Their metamorphosis was accelerated by several months. The appearance of the reflex was correspondingly accelerated, although it was again somewhat delayed relative to the emergence of the forelimbs, following the latter after 2 days in one animal, and after 4 days in the other.

3. *Bufo fowleri*. Thyroxin treatment was attempted with fifty *Bufo fowleri* tadpoles at a time when their hind limbs had just started to grow rapidly. Metamorphosis was accelerated by 2 to 4 days as compared with a control group, and the appearance of the corneal reflex was equally advanced.

4. *Hylidae*. Thirty hylid tadpoles with small, functional hind limbs were subjected to thyroxin treatment. As with *Bufo*, the acceleration was slight, and the onset of the reflex was not shifted with respect to the other metamorphic changes.

Thus it appears that thyroxin treatment accelerates both general metamorphosis of the anurans and the time of onset of the reflex, but it accelerates the latter to a slightly less extent than it does the former. To obtain further evidence relating the onset of the reflex to other metamorphic events, two hypophysectomized *Rana pipiens* tadpoles of 45 weeks were treated with increasing doses of thyroxin. After 19 days the dorsal skin spots appeared. The thyroxin concentration at this time was 1×10^{-7} . At 21 days, with the hind legs grown to about three-fourths of the length of the body, the corneal reflex appeared. By this time the tail fins had resorbed. At 30 days the forelimbs emerged, and at 35 days the thyroxin concentration was raised to 3×10^{-7} , at which level it was maintained until the death of the animals on the forty-seventh day. Before death occurred the animals had developed a skin pattern with prominent dorsal rings on a green background. The eyes bulged slightly and had well developed nictitating membranes. The threshold for the corneal reflex had dropped considerably, but it was not quite so low as in normal adults. The mouth had begun to broaden, after losing the horny beak, but the upper jaw was transforming at a faster rate than

the lower jaw. The tail musculature remained, and the gills, which had failed to become resorbed, were protruding far out of the much enlarged opercular perforations through which the forelegs had emerged. The abnormal metamorphosis, which can probably be attributed, at least in part, to the thyroxin concentrations used, may account for the 9-day lapse between the onset of the reflex and the emergence of the forelimbs, which compares with only a 3- or 4-day lapse in normal metamorphosis. Even so, however, this experiment strikingly illustrates the close relationship between the onset of the reflex and metamorphic climax in the anurans.

DISCUSSION

The corneal reflex is elicited by the stimulation of the cornea or the adjacent skin. Such stimulation results in the contraction of the retractor bulbi muscle, which acts by pulling the bulb into the orbit and lifting the nictitating membrane. As the bulb is retracted, the eyelids passively fold over it.

The claims of Petersen ('24), Beers ('29), Stone ('30, '38), Stone and Ussher ('27), and Stone, Ussher and Beers ('37), indicating that the corneal reflex first appears at metamorphosis or immediately before, have been confirmed for anurans and for a certain proportion of the maximally fed urodeles. All species of anurans which were studied normally displayed the reflex for the first time in a period from a few days before to a few days after the forelimbs emerged, that is, at the beginning of metamorphic climax. In only a few instances did the reflex appear earlier than 5 days before forelimb emergence. In view of the evidence presented here, the claim of Giesbrecht ('25), that the reflex is obtainable in *Rana esculenta* larvae whose hind limbs are but a few millimeters long, cannot be accepted without reinvestigation. In well fed *Amblystoma* the corneal reflex usually appears from 3 weeks to a few days before the beginning of gill resorption. By controlling the nutritional level of the larvae it is possible to alter the time of onset of the reflex. Thus in very poorly fed larvae the reflex is delayed in appearance, but because

metamorphosis is delayed even more, the reflex may appear several months before metamorphosis, as against only 3 weeks before metamorphosis in well fed animals. Because of the great extension of the larval period in inanition, the reflex is present for a maximum of 50 to 60% of this period, contrasting with its presence for only 25% of the larval period in well fed animals. The situation is materially different in anurans, in which the reflex never appeared until just a few days before the emergence of the forelimbs, even when the larval period was extended to more than three times its usual length by partial starvation of the tadpoles. These results suggested that if by hypophysectomy the larval condition were to be perpetuated, the anurans would permanently lack the reflex, whereas the urodeles would develop it. This possibility was tested. The oral anlage of the hypophysis was removed in the tail bud stage of *Amblystoma punctatum*, *A. tigrinum* and *Rana pipiens* embryos. Eleven weeks later the hypophysectomized *Amblystoma* displayed the reflex. In urodeles, apparently, metamorphosis is not a prerequisite for the development of the reflex, for these animals did not subsequently metamorphose. Hypophysectomized *Rana*, on the other hand, failed to display the reflex even after 80 weeks, some six times longer than their usual larval period.

The reciprocal experiment, acceleration of metamorphosis, was also performed. The results depended upon the amount of acceleration produced by the thyroxin treatment. If metamorphosis was advanced less than 2 weeks, the reflex was equally advanced in its onset. If metamorphosis was advanced 3 weeks, the onset of the reflex was advanced by only about 2 weeks, that is, instead of appearing some 4 days before the emergence of the forelimbs, the reflex first appeared 2 or 3 days after the forelimb emergence. When metamorphosis was advanced by as much as 5 weeks, the death of the animals occurred 3 or 4 days after the emergence of the forelimbs, without the reflex having ever appeared. The changes responsible for the reflex are apparently less accelerated by

thyroxin than are those responsible for other metamorphic transformations.

In *Amblystoma* larvae kept at low nutritional levels it has been noted that the reflex appears later than in well fed larvae, but at a relatively earlier stage with respect to the total length of the larval period. In these poorly fed animals the reflex is first evidenced as a rotation of the eyeball, which is gradually replaced by retraction. In either urodele or anuran larvae which fail to display the reflex, electrical stimulation of the retractor bulbi muscle or of the abducens nerve produces a contraction of the muscle, which, owing to the posterior position of its point of origin, results in a rotation of the bulb. In more mature animals the point of origin of the muscle has shifted toward the midline; the muscle fibers are then parallel to the medio-lateral axis of the eyeball, and contraction of the muscle effects pure retraction. In the poorly fed animals this shift in the muscle origin from a lateral to a medial position occurs several months before metamorphosis, whereas in the well fed animals with a much shorter larval period the shift is not completed until 1 or 2 weeks before metamorphosis.

In each species studied the area from which the reflex can be elicited appears to have a characteristic extent, although individual variations occur. When the reflex is first elicitable in urodeles, the area usually includes only the cornea, or a fraction of it, or some small fraction also of the lids. This area then gradually spreads, until at metamorphosis it reaches its maximum extent. As such it is preserved in the adult. In poorly fed larvae the spread of the reflexogenous area is slower than in the well fed ones, but as the larval period is prolonged, the reflexogenous area continues to spread even beyond the normal limits, until in some urodeles it includes the entire dorsal surface of the head. In anurans it is impossible, by any means, to increase the reflexogenous area.

Histological study of the cornea in the midlarval, or even the embryonic, period reveals the presence of nerve fibers, and tactile stimuli applied to the cornea of animals in these stages produce swimming responses, indicating that these sensory

nerve fibers are functioning. Dissection of the orbit of mid-larval animals shows that the retractor bulbi muscle is present, and electrical stimuli applied to the muscle or its nerve result in contractions, indicating that the muscle is functionally innervated. Since the corneal reflex cannot be elicited at this time, and since this failure cannot be attributed to insufficiency of the peripheral components of the reflex, apparently the central mechanism of the reflex has not yet developed. Presumably in anurans its development depends upon the metamorphosing stimulus (release of colloid from the thyroid gland), whereas in urodeles it is independent of this. In urodeles, seemingly, the mechanisms bringing about the onset of the corneal reflex and the onset of metamorphosis are, at least to some extent, independent, being normally associated at metamorphic climax only by coincidence. The association of the onset of the reflex with metamorphic climax is not particularly close, for even in well fed animals the onset of the reflex may precede metamorphosis by as much as 3 weeks or by as little as 2 days. The dissociation of the reflex still further from metamorphosis by inanition and by hypophysectomy, however, make the above conclusion evident. In urodeles metamorphic changes are less radical and come about more gradually than in anurans, consequently metamorphic climax in the former group is far less striking than it is in the latter. Etkin and Huth ('39) have put forward evidence that in *Rana* a premature activation of the thyroid gland results when that organ is in close proximity to the pituitary body, and a premature metamorphosis occurs. The same experiments, when repeated in *Amblystoma punctatum* (Lynn, '40) yielded negative results, as neither thyroid stimulation nor premature metamorphosis occurred. This is further evidence of differences in the method of metamorphosis between urodeles and anurans.

CONCLUSIONS

1. The corneal reflex is not elicitable in larval amphibia, generally, but is in the adult.

2. The reflex is elicited by stimulation of the cornea or of the adjacent skin. It consists of a withdrawal of the bulb into the orbit by the retractor bulbi muscle, a lifting of the nictitating membrane, and a passive closure of the lids.

3. The innervation of the cornea functions, and the retractor bulbi muscle and its motor innervation are capable of functioning in the midlarval stages, long before the reflex can be elicited by adequate sensory stimulation of the cornea. The sudden appearance of the reflex, therefore, appears to be dependent upon changes in the brain centers concerned with the reflex.

4. In well fed animals the reflex generally appears during metamorphic climax, or shortly before. In urodeles the reflex may precede metamorphosis by 3 weeks, in anurans by 1 week. Each species has its own period for the onset of the reflex. In *Bufo fowleri*, *Rana pipiens*, and *R. catesbiana* it is before the emergence of the forelegs, while in *Hyla crucifer*, *H. versicolor*, and *Pseudacris triseriata* it is after foreleg emergence and during the period of tail resorption. Inanition slows growth, but in anurans the reflex still appears in normal relationship to other metamorphic changes. In urodeles, however, the period of onset of the reflex varies with the rate of growth. The corneal reflex is inhibited in its development by inanition, but to a lesser extent than the body generally. Poorly fed animals may develop the reflex as much as 5 months before metamorphosis.

5. Hypophysectomy perpetuates the larval condition, and in anurans prevents the appearance of the reflex. In urodeles, however, the reflex still develops.

6. If anuran tadpoles are treated with thyroxin, the onset of the reflex is advanced, but somewhat less than is metamorphosis in general. In *Rana*, for example, the reflex appears after, rather than before, the emergence of the forelimbs.

7. The reflex exhibits temporal and spatial summation, both in developmental and adult phases.

8. When the reflex first appears it is elicitable from only the cornea, or a restricted portion of the cornea, but the re-

flexogenous area then expands, until metamorphosis freezes the size of the area. The extent of the reflexogenous area varies with the species; in all of the anurans tested it is limited to the cornea, lids, and skin between the nostril and the eye.

In urodeles it extends from the nostril to a point back of the eye, the distance differing in the various species, but increasing with larval age. Poorly fed larvae, with a long larval period, have the largest reflexogenous area.

9. The differences between urodeles and anurans with respect to the fixity of the time of onset of the reflex and the spread of the reflexogenous area furnish further evidence that metamorphosis differs somewhat in the two groups.

LITERATURE CITED

- ADOLPH, E. F. 1931 The size of the body and the size of the environment in the growth of tadpoles. *Biol. Bull.*, vol. 61, pp. 350-375.
- BEERS, D. N. 1929 Return of vision and other observations in transplanted amphibian eyes. *Proc. Soc. Exp. Biol. and Med.*, vol. 26, pp. 477-479.
- BLOUNT, R. F. 1935 Size relationships as influenced by pituitary rudiment implantation and extirpation in the urodele embryo. *J. Exp. Zool.*, vol. 70, pp. 131-185.
- BODIAN, D. 1936 A new method for staining nerve fibers and nerve endings in mounted paraffin sections. *Anat. Rec.*, vol. 65, pp. 89-97.
- 1937 The staining of paraffin sections of nervous tissues with activated protargol. The role of fixatives. *Anat. Rec.*, vol. 69, pp. 153-162.
- COGHILL, G. E. 1929 *Anatomy and the Problem of Behavior*. The University Press, Cambridge.
- D'ANGELO, S. A., S. A. GORDON, AND H. A. CHARIPPER 1941 The role of the thyroid and pituitary glands in the anomalous effect of inanition on amphibian metamorphosis. *J. Exp. Zool.*, vol. 87, pp. 259-277.
- ECKER, A., R. WIEDERSHEIM, UND E. GAUPP 1896 *Die Anatomie des Frosches*. Braunschweig.
- ETKIN, W. 1935 The mechanisms of anuran metamorphosis. I. Thyroxine concentration and the metamorphic pattern. *J. Exp. Zool.*, vol. 71, pp. 317-340.
- ETKIN, W., AND T. HUTH 1939 A thyrotropic field effect in the tadpole. Part I. *J. Exp. Zool.*, vol. 82, pp. 463-495.
- FRANCIS, E. T. B. 1934 *The Anatomy of the Salamander*. The Clarendon Press, Oxford.
- GIESBRECHT, E. 1925 Beiträge zur Entwicklung der Cornea und zur Gestaltung der Orbitalhöhle bei den einheimischen Amphibien. *Zeit. f. Wiss. Zool.*, Bd. 124, S. 305-359.
- HARMS, W. 1923 Brillen bei Amphibienlarven. *Zool. Anz.*, Bd. 56, S. 136-142.

- KOLLOS, J. J. 1941 The development of the corneal reflex in Amphibia. *Anat. Rec.*, vol. 79, suppl. no. 2, p. 38.
- KOPPÁNYI, T. 1923 Die Replantation von Augen. II. Haltbarkeit und Funktionsprüfung bei verschiedenen Wirbeltierklassen. *Arch. mikr. Anat. u. Entwmech.*, Bd. 99, S. 15-42.
- LYNN, W. G. 1940 Results of transplantation of the pituitary anlage to the thyroid region of *Amblystoma*. *Biol. Bull.*, vol. 79, p. 361.
- PETERSEN, H. 1924 Berichte über Entwicklungsmechanik. I. Entwicklungsmechanik des Auges. *Ergeb. d. Anat. u. Entw. Gesch.*, Bd. 25, S. 623-660.
- PIATT, J. 1934 Morphogenesis of the cranial muscles of *Amblystoma punctatum*. *J. Morph.*, vol. 63, pp. 531-587.
- ROMANO, M. 1936 Le modificazioni dell'occhio degli Anuri durante la metamorfosi. *Arch. Ital. d'Anat. e di Emb.* T. 36, pp. 433-465.
- SMITH, P. E. 1913 The effect of hypophysectomy in the early embryo upon the growth and development of the frog. *Anat. Rec.*, vol. 11, pp. 57-64.
- 1920 The pigmentary, growth and endocrine disturbances induced in the anuran tadpole by the early ablation of the pars buccalis of the hypophysis. *Am. Anat. Mem.*, No. 11, pp. 1-151.
- SMITH, P. E., AND I. P. SMITH 1922 The repair and activation of the thyroid in the hypophysectomized tadpole by the parenteral administration of fresh anterior lobe of the bovine hypophysis. *J. Med. Res.*, vol. 43, pp. 267-284.
- STONE, L. S. 1930 Heteroplastic transplantation of eyes between the larvae of two species of *Amblystoma*. *J. Exp. Zool.*, vol. 55, pp. 193-261.
- 1938 Return of vision and other observations in grafted vertebrate eyes. *Am. J. Ophth.*, vol. 21, pp. 1-6.
- STONE, L. S., AND N. T. USSHER 1927 Return of vision and other observations in replanted amphibian eyes. *Proc. Soc. Exp. Biol. and Med.*, vol. 25, pp. 213-215.
- STONE, L. S., N. T. USSHER, AND D. N. BEERS 1937 Reimplantation and transplantation of larval eyes in the salamander (*Amblystoma punctatum*). *J. Exp. Zool.*, vol. 77, pp. 13-48.
- TWITTY, V. C., AND H. A. ELLIOT 1934 The relative growth of the amphibian eye, studied by means of transplantation. *J. Exp. Zool.*, vol. 68, pp. 247-291.
- TWITTY, V. C., AND J. L. SCHWIND 1931 The growth of eyes and limbs transplanted heteroplastically between two species of *Amblystoma*. *J. Exp. Zool.*, vol. 59, pp. 61-86.
- WINDLE, W. F. 1940 Physiology of the Fetus; Origin and Extent of Function in Prenatal Life. W. B. Saunders Co., Philadelphia and London.

EXPERIMENTAL STUDIES ON THE DEVELOPMENT OF THE CORNEAL
REFLEX IN AMPHIBIA. II. LOCALIZED MATURATION OF THE RE-
FLEX MECHANISM EFFECTED BY THYROXIN-AGAR IMPLANTS
INTO THE HINDBRAIN

(Three figures)

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IN A previous study on the develop-
ment of the corneal reflex in Am-
phibia (Kollros, 1942a), it was re-
ported that in all anuran tadpoles the on-
set of the reflex coincided approximately
with metamorphic climax. The reflex al-

ways appeared within a period of from a
few days before to a few days after the
emergence of the forelimbs from the
branchial chamber. While in urodeles

¹ This research was done under the direction of
Dr. Paul Weiss in partial fulfilment of the require-

the reflex appears even in hypophysectomized animals, which never metamorphose, it is never elicitable in hypophysectomized anurans unless they are artificially stimulated to metamorphose through thyroid administration. In these artificially metamorphosed animals, as in normal ones, the first appearance of the reflex precedes the emergence of the forelimbs by only a few days. This very close association of the initial appearance of the reflex with metamorphosis in anurans suggests that the reflex center is dependent for its maturation upon the metamorphosing agent, thyroid hormone.

Modifications of behavior induced by hormone treatment have received considerable attention, much of which has been directed toward studies of the relationship between sex hormones and sexual behavior.² Stone (1927), for example, demonstrated that the castration of rats led to a dwindling of their sexual aggressiveness until finally they no longer copulated. Similar studies on rabbits yielded similar results (Stone, 1932). In bilaterally ovariectomized fowl, in which grafts of juvenile testes had been established, Domm (1929) demonstrated crowing, fighting with males, and attempts to tread. Ball (1937) noted that female rats injected with testosterone propionate exhibited masculine behavior. Allee, Collias, and Lutherman (1939) injected normal hens with testosterone propionate. They noted an increase in aggressiveness, initiation of crowing, and, in three hens, initiation of courtship behavior. Shortly

after the treatments stopped, these changes disappeared.

Hormones other than those of the sex glands have also been shown to influence the central nervous system and behavior; for example, prolactin induces broodiness in the jewel fish (Noble, Kumpf, and Billings, 1936) and in the domestic fowl (Riddle, Bates, and Lahr, 1935). Thyroid hormone has a marked influence upon the human nervous system. In hyperthyroid states the individual is irritable; in hypothyroid conditions, sluggish; the two conditions also have opposite effects upon the sympathetic nervous system, as revealed by vasomotor and peristaltic activity (Lerman, 1941). Recently Ross and Schwab (1939) discovered that the rate of the alpha rhythm of brain waves obtained from the posterior part of the skull, as recorded by the electroencephalogram, is low in myxedema and returns to normal upon thyroid administration. The correlation between the alpha rate and the state of metabolism was found to be good.

Hormones are capable of modifying not only certain established behavioral traits or of initiating new ones in adults but also of hastening the onset of as yet undeveloped behavioral traits in the young. Domm (1937) injected male chicks with hebin, beginning at the age of 2-4 days. After six injections the chicks crowed, and in some cases they were treading by the thirteenth day. Shortly after the injections were stopped, these adult masculine traits disappeared. A similar precocious establishment of adult traits is brought about by thyroid administration to tadpoles. This results in a premature metamorphosis (Guder-natsch, 1912), including the associated behavioral changes incident to life on land. Among these is the lid-closure, or corneal, reflex.

ments for the degree of Doctor of Philosophy. It has been aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. A preliminary report appeared in *Proc. Soc. Exper. Biol. and Med.* (Kollros, 1942b).

² See Stone (1939) for an extensive review.

It was the purpose of the present experiments to determine whether or not thyroid hormone is directly responsible for the maturation of the center for the lid-closure reflex. Since thyroid treatment of the animal as a whole results in general metamorphosis, it cannot be determined whether the maturation of the center depends directly upon the hormone or whether it depends secondarily upon hormone-induced changes in some other structures, much as the development of the tympanic membrane, for example, depends upon the formation of the annular tympanic cartilage (Helff, 1928). To prove a direct effect of thyroid hormone upon the reflex mechanism it would be necessary to treat the reflex center alone; if the reflex were to appear prematurely, unaccompanied by any other metamorphic changes, the efficacy of the hormone in bringing about the maturation of the center would be demonstrated. It might be possible to obtain such local effects by implanting pellets of agar, previously soaked in a thyroxin solution, into the fourth ventricle of the medulla oblongata in the region of the reflex center. The local high concentration of thyroxin could be expected to speed the maturation of the reflex mechanism. Implants of thyroxin-soaked agar were first used by Hartwig (1940) to induce local metamorphosis of the dorsal skin and the tail fin of *Salamandra* larvae.

MATERIALS AND METHODS

Tadpoles of *Rana pipiens*, nearly all of which were in mid-larval stages or older, were used. The tadpoles were kept in individual finger bowls and were fed on boiled lettuce. Tactile stimuli were applied with a series of fibers. In all cases the fiber was applied with its side rather than its tip. The stiffness of the fibers was graded according to the method de-

scribed by Weiss (1942). The tip of the fiber was held against the edge of a pan of an analytical balance, and the counterweight necessary to bend it by about 15° was determined. The values for fibers 1-4 were 3, 18, 57, and 300 mg., respectively. Four modes of stimulation were used: (1) single-point contact, (2) repeated touch to the same spot (temporal summation), (3) single stroke over a larger area (spatial summation), and (4) repeated strokes (temporal and spatial summation). Stimuli were applied to the cornea alone in these tests.

All stimulations were performed under the low power (20X) of a wide-field binocular dissecting microscope. The tadpole remained under water and was unrestrained during stimulation. By the term "metamorphic climax" is meant the period of greatest metamorphic activity, the start of which in anurans can be arbitrarily set as the time of forelimb emergence from the branchial chamber (hereinafter referred to as "f.l.e."). The period immediately preceding metamorphic climax will be termed the "premetamorphic" period or stage.

The tadpoles were anesthetized in a 2 per cent solution of ethyl urethane and were operated upon in amphibian Ringer's solution. A lengthwise cut of 3 or 4 mm. was made through the skin in the midline just dorsal to the medulla oblongata. The edges of the cut were pulled back by small retractors. In the older tadpoles a thin plate of cartilage lies just above the medulla, while in the younger ones the cartilage has not yet formed and only a thin layer of connective tissue lies above the medulla, stretching across between the two lateral masses of muscle. After the cartilage or connective tissue was removed and the muscles on either side pulled back, the chorioid plexus could be seen roofing over the fourth ven-

tricle. A slit about $\frac{3}{4}$ mm. long was made in the chorioid plexus; the blood from this wound was blown away by a jet of Ringer's solution from a small pipette. The bleeding usually stopped in from 10 to 20 seconds. A thyroxin-soaked agar pellet was then introduced into the fourth ventricle through the slit previously made. The overlying tissue was permitted to fall into place, and the edges of the skin were apposed. A small clot usually formed under the edges of the cut. Shortly after recovering from the anesthetic, the tadpole was transferred into fresh well water.

Thyroxin crystals (E. R. Squibb & Sons) were dissolved in slightly alkaline water to furnish the stock solutions in concentrations of 1:500 and 1:1,000. The agar pellets were cut from a larger block of agar. The length of the pellets was never greater than 0.5 mm., while in width and thickness they ranged from 0.1 to 0.5 mm. These pellets were placed in the thyroxin solution for about $\frac{1}{2}$ hour. Shortly before being implanted they were placed on a dry glass plate and permitted to harden slightly. The harder surface prevented their being crushed in subsequent manipulations. In some cases fragments (usually several follicles) of fresh rat thyroid gland were substituted for the thyroxin-soaked agar pellets.

EXPERIMENTAL

In Part I of this study (Kollros, 1942a) twenty *R. pipiens* tadpoles had been treated by adding thyroxin to the water (diffuse treatment), with the effect that metamorphosis was markedly advanced. The results of this experiment are shown graphically in Bars II and III of Figure 1. Normally the initial appearance of the reflex precedes f.l.e. by an average of 4 days, while in the thyroxin-accelerated

animals, whose metamorphosis had been advanced by as much as 2 or 3 weeks, the onset of the reflex preceded f.l.e. by only 1 or 2 days. If metamorphosis was advanced by about $3\frac{1}{2}$ weeks or more, the onset of the reflex followed f.l.e. by from 1 to 4 days, and in some animals which died on the fifth day after f.l.e. it failed to appear at all. If thyroid hormone does influence the maturation of the reflex center directly, its presence in supernormal concentration, diffusely applied, certainly advances the maturation of the center somewhat less than it does metamorphic changes in body structures, as, for example, the opercular perforation, skin-pigment changes, tail resorption, and so on.

As was indicated before, the implantation of thyroxin-soaked agar pellets into the fourth ventricle might be expected to produce a premature appearance of the lid-closure reflex, owing to the local accelerating action of the hormone on the maturation of the reflex mechanism. In addition, as the thyroxin enters the blood stream by diffusion, a general effect on the animal as a whole might be expected. Because of the lower concentration of the thyroxin in the blood as compared to that in the immediate vicinity of the graft, including the reflex center, the general metamorphic progress of the animal could be expected to be slower than that of the center.

As the onset of the reflex normally precedes f.l.e. by an average of 4 days (Fig. 1, Bar I), any appearance of the reflex preceding f.l.e. by an average of more than 4 days would indicate a stronger local effect. Actually, since diffuse thyroxin treatment displaces the onset of the reflex to a later stage in the metamorphic sequence (Fig. 1, Bars II and III) and as the general influence of the implant on the body generally would tend to do likewise, the relative acceleration of the on-

set of the reflex as compared to f.l.e. will be even greater than recorded.

Series A.—In this series either agar pellets previously soaked in a 1:1,000 solution of thyroxin or fresh rat thyroid fragments were implanted in the fourth ventricle of *R. pipiens* tadpoles at various mid-larval stages. The capacity of rat thyroid implants to stimulate metamorphosis in *Rana* had already been demonstrated (Little, 1929), and the fate of such grafts in *Triturus* had been studied by Idzkowsky (1932). The potency of implanted mouse thyroid in stimulating molting in thyroidectomized *Triturus* had also been tested and the replacement of the implants by host tissue observed (Adams and Merwin, 1939). In the present series only fiber 2 was used as a test stimulus. The first appearance of the reflex was determined by stroking the cornea repeatedly, this being the most effective stimulus. Only after the reflex was established were the other modes of stimulation employed.

Twenty-nine control animals were used. In these the reflex preceded f.l.e. by from 0 to 10 days, or by an average of 4.1 days. This compares very well with earlier observations, in which the period before f.l.e. was given as 4 days (Kollros, 1942a). This group and the control group of Series C are indicated by Bar I in Figure 1.

Thirty-three animals with implants in the fourth ventricle first displayed the reflex from 1 to 18 days (average 10.5 days) prior to f.l.e. This treated group can be subdivided according to the stage at the time of implantation. Twelve animals which had received the implant at a stage when no more than three toes were indicated on the hind limb developed the reflex at the normal time or only slightly earlier (Fig. 1, Bar IV). Most of these animals showed an initial phase of meta-

morphic stimulation; but before the reflex appeared they entered a period of metamorphic stasis of about 2 weeks' duration, due apparently to exhaustion of the thyroid hormone content of the implant. These animals had the appearance typical of thyroid-treated tadpoles.

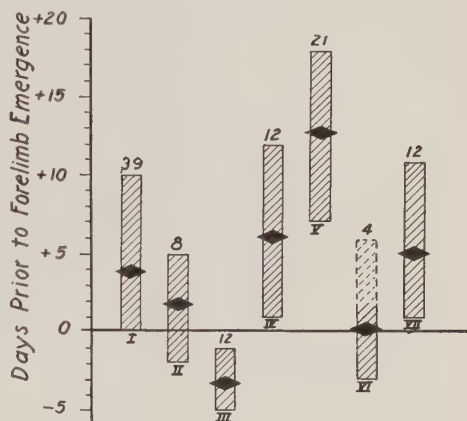


FIG. 1.—The bars represent the time range in days by which the onset of the corneal reflex preceded (or followed) the emergence of the forelimbs. The diamond indicates the average for each group. The number above the bar indicates the number of animals in each group. I, normal controls; II, thyroxin added to water (concentrations less than 1:1,000,000); III, thyroxin added to water (concentrations above 1:1,000,000). IV, agar implants (previously soaked in 1:1,000 thyroxin) in the fourth ventricle of young mid-larval tadpoles; V, similar implants in older mid-larval tadpoles; VI, agar implants (previously soaked in 1:500 thyroxin) in the abdomen of older mid-larval tadpoles; VII, similar implants in the fourth ventricle of older mid-larval tadpoles. The animals represented by Bars VI and VII are from different stocks from those of the other experimental groups.

They remained considerably shorter than their controls, and, in contrast to the latter, their bodies were constricted just posterior to the gills. Development of the hind limbs to an early functional phase had been accelerated and then suddenly terminated. In a few cases incipient changes in pigmentation were noted. Subsequent development to metamorphosis was approximately normal. Little

(1929) observed the same phenomenon of stimulation followed by metamorphic stasis in *R. catesbiana* tadpoles which had received large abdominal implants of rat thyroid glands.

The remaining twenty-one animals of this series were somewhat older at the time of implantation (all five digits indicated on the hind-limb bud). As these animals were more mature at the time of implantation than were those of the previous group, the hormone content of the implant seemed to have been sufficient to permit them to pass through metamorphosis without an intervening stasis period. In many animals, however, the last portion of the period prior to f.l.e. was passed through at a normal rate rather than an accelerated one. In this second group of animals the reflex never appeared less than 7 days prior to f.l.e., with an average period of 12.9 days (Fig. 1, Bar V). The maximum time interval between the onset of the reflex and f.l.e. was 18 days, as compared to 10 days for the controls. The length of the period between implantation and the onset of the reflex was 12 days, on the average, whereas about 20 days would have had to elapse had the tadpoles been subjected to thyroxin treatment at a concentration which would have advanced their metamorphosis to an equal degree.

Ten of the twenty-one animals developed the reflex asynchronously on the two sides. Whereas in the control group the two eyes were never more than 1 day apart in first displaying the reflex, some of the experimental animals showed a difference between the two eyes of as much as 7 days. The asymmetry can be ascribed to an asymmetrical position of the implant, the side of the brain wall nearer the graft being subjected to a somewhat higher hormone concentration than the opposite side. This interpreta-

tion is borne out by two cases studied in microscopic sections, in both of which the unilateral precocity of the reflex was found to correspond to proximity between the implant and one brain wall (Fig. 2).

As a further check on the local influence of the implants, four tadpoles received implants of thyroxin-soaked pellets in one of the lateral forebrain ventricles. The metamorphosis of all the animals was hastened, but in none did the corneal reflex appear early in the sequence of metamorphic changes. Figure 3 shows a section of the forebrain with the implant.

Series B.—Agar pellets previously soaked in a 1:1,000 thyroxin solution were implanted in the fourth ventricles of seven tadpoles about 2 weeks prior to the expected onset of the reflex. Tactile stimuli were applied as in Series A. In three animals the reflex appeared overnight, and in the four others it appeared by the second day after implantation. This treatment also approximately halved the expected interval to f.l.e. Even so, in two of the cases, the reflex preceded f.l.e. by as much as 8 days. Such speedy appearance of the reflex suggests that the reflex mechanism was essentially mature at the time of operation, merely requiring thyroid activation to be set off.

Series C.—In this third series only agar implants were used. The tadpoles, all of which were in the later mid-larval stage, were divided into four groups. In Group I were placed ten untreated controls. In Group II there were four animals, each of which had had a thyroid-free agar pellet implanted in the fourth ventricle. This group provided a control to determine possible effects of the operative procedure. Four animals (Group III) received abdominal implants of agar pellets previously soaked in a 1:500 thy-

roxin solution. This group provided a control of the generalized metamorphosing effects of the implants. In twelve animals (Group IV) similarly soaked pellets were implanted in the fourth ventricle.

the reflex would be elicitable somewhat earlier than in the control group of Series A. On the contrary, it appeared later, preceding f.l.e. by from 1 to 4 days (average of 2.7 days) in the untreated controls

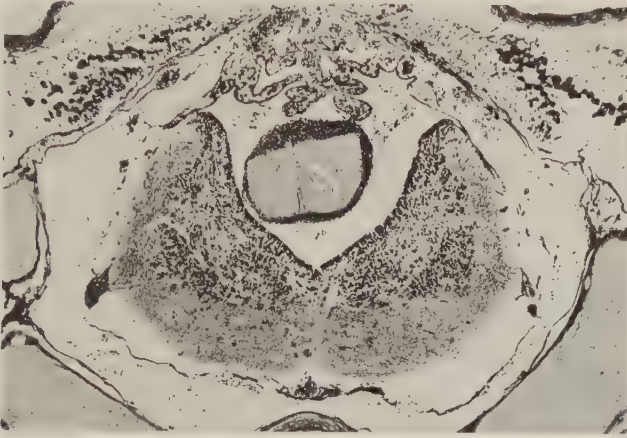


FIG. 2.—Thyroxine-soaked agar implant in the fourth ventricle. The implant has become encapsulated. Protargol-silver impregnation. $\times 41$.

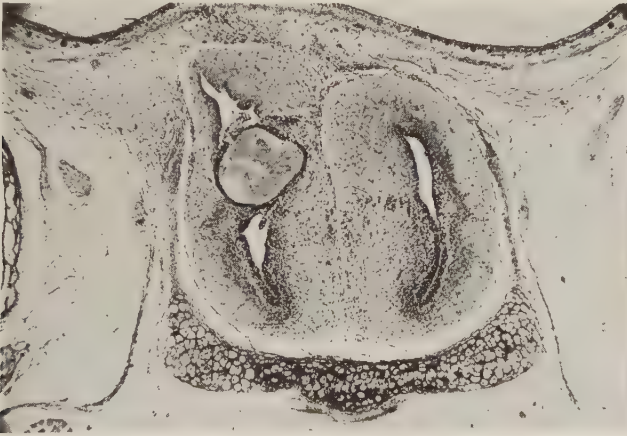


FIG. 3.—Thyroxine-soaked agar implant in the forebrain (right lateral ventricle). Ehrlich's haematoxylin-eosin. $\times 28$.

In this series the appearance of the reflex was tested by strokes of fiber 4, in contrast to the previous series in which fiber 2 had been used. As stronger stimuli can be administered with fiber 4 than with fiber 2, it was to be expected that

(Group I) and by an average of 3.8 days in the operated controls (Group II). These results suggest that in the present stock of tadpoles the time differential between the onset of the reflex and f.l.e. is consistently less than in Series A.

Groups III and IV, which had received implants of thyroxin-soaked agar, developed at equal rates, each about twice as fast as normal. They displayed the usual features of intense thyroid stimulation, such as very rapid growth of the forelimbs and hind limbs, precocious appearance of the opercular perforation and emergence of the forelimbs, very rapid pigment changes, constriction of the body just behind the gills, and a disproportionate bulging of the eyeballs. Of the four animals with abdominal implants, one first displayed the reflex 6 days prior to f.l.e., while the others displayed it 1, 1, and 3 days after f.l.e. (Fig. 1, Bar VI). The aberrant behavior of the first animal is not readily explained.

In Group IV the implant could act locally upon the reflex center, as well as exert its general metamorphosing effects by way of the circulation. The appearance of the reflex preceded f.l.e. by from 1 to 11 days, or by an average of 5.2 days (Fig. 1, Bar VII). Because of the very great metamorphic acceleration of the body generally, the local effects of the implant were masked. Even so, the local action produced the 5-day lapse between the results shown in Bars VI and VII. If the single aberrant animal of Group VI were to be left out of consideration, the time lapse between the two groups would be 7 days.

DISCUSSION

In the anurans previously studied it had been impossible to elicit the corneal reflex prior to the period of metamorphic climax, despite the fact that both the afferent and the efferent portions of the reflex arc had been shown to be functional, or at least capable of functioning (Kollros, 1942a). This evidence suggested, first of all, that the failure of the reflex to be elicited in younger larvae might be ascribed to some deficiency of the reflex

center in the larval hindbrain and, second, that this deficiency was normally overcome by the action of the metamorphosing agent, thyroid hormone. Attempts to bring about a premature maturation of the reflex mechanism through the use of thyroid hormone were made in two different ways: by thyroxin administration to the entire animal and by local implants of thyroxin-soaked agar pellets or rat thyroid tissue to the vicinity of the reflex center in the medulla oblongata. The addition of thyroxin to the water in which the tadpoles were kept advanced the metamorphosis of the whole animal markedly, but it failed to produce an appearance of the reflex earlier in the metamorphic sequence than was normal. In fact, the stronger the thyroxin concentration, the farther back in the sequence the onset of the reflex was pushed. Instead of first appearing 4 days before the emergence of the forelimbs from the branchial chamber (f.l.e.), as in the controls, the reflex first appeared from 2 days before to 4 days after f.l.e.

In contrast to the results of diffuse treatment, local application of thyroxin in high concentration to the tadpole hindbrain in the vicinity of the reflex center did produce an appearance of the reflex earlier in the metamorphic sequence than was normal. This was in addition to (i.e., superimposed upon) the advance in metamorphosis of the whole animal, which was presumably caused by the accumulation in the blood stream of some of the thyroxin from the implant.

Since, as has just been shown, acceleration of general metamorphosis through diffuse thyroxin treatment hastens the onset of the reflex less than it does other metamorphic changes, the nonlocalized effects of the thyroxin-agar implants could be expected to have the same result. Although the local effect of the im-

plant would advance the maturation of the reflex center, the diffuse effects would advance the metamorphosis of the rest of the body, thus decreasing the apparent influence of the implant upon the center, particularly since the center is less responsive to thyroxin than are other body structures. In diffuse treatment it was seen that the more concentrated the thyroxin solution, the greater was the disparity between the metamorphic advance of the reflex center and that of the rest of the body (Fig. 1, Bars I, II, III). A similar disparity was noted in the animals receiving the local implants. Those animals receiving pellets previously soaked in the 1:1,000 thyroxin solution showed a moderate metamorphic advance; but, because of the local influence of the implant, a still greater advance in the time of onset of the reflex was noted (onset of the reflex preceded f.l.e. by an average of 12.9 days). In those animals receiving implants previously soaked in the 1:500 thyroxin solution, the thyroxin content of the blood apparently became high enough so that body metamorphosis was greatly advanced, while the less responsive reflex center, although subjected to a still higher thyroxin concentration, did not have its metamorphosis advanced significantly more than did the body generally (onset of the reflex preceded f.l.e. by an average of 5 days). When compared to its control group (Ser. C, Group III), however, the advance is seen to be significant. Apparently the generalized effects of the implants mask their local influence upon the reflex center, at least in part. This is particularly true when the implants have been soaked in strong thyroxin solutions.

Because the experiments of Series A and C were carried out on two different stocks of tadpoles and because the sizes

of the implants (and thus of the amount of thyroxin contained in the implant) varied, the figures quoted above may not be of the proper magnitude, although the differences between the results of Series A and C are probably significant. This in turn suggests that some still lower concentration of thyroxin might be found which, although capable of bringing the reflex center to functional maturity, would be too low to stimulate general metamorphosis appreciably. A period of more than 13 days would then be expected to intervene between the first appearance of the reflex and f.l.e.

The implantation of the thyroxin-agar pellets in the fourth ventricle in the vicinity of the center for the lid-closure reflex did not always induce a shift in the phase of metamorphosis during which the reflex first appeared. If the tadpoles were still so young at the time of implantation as to lack some of the digits of the hind limb, the first appearance of the reflex was premature only slightly as compared to that in untreated control animals. Apparently the reflex mechanism was not yet prepared to respond to thyroxin treatment. Tadpoles scarcely a week older than those just discussed, and receiving similar implants, do display the reflex prematurely (Ser. A). Apparently the reflex center has become so modified in that interval as to be able to mature under the influence of the thyroxin. In these animals the average length of the period between implantation and the first appearance of the reflex was about 12 days, while with diffuse thyroxin acceleration approximately 20 days would have been required. By using still older animals it was possible to elicit the reflex from 12 to 36 hours after implantation, whereas normally 7-12 days would have had to elapse. These results suggest that the development of the reflex mechanism to the

functional phase progresses in three stages: In the early larval and mid-larval stages the reflex mechanism is self-differentiating within the brain centers independent of hormone action (Weiss, 1941); in the later mid-larval period the center comes under the influence of thyroid hormone and through its action is brought to maturity; finally, when the metamorphic stage is reached, the hormone precipitates the setting-into-action of the reflex mechanism. As is suggested by Series B, the mechanism is ready for action in the premetamorphic period but would normally be delayed in its appearance until a few days before f.l.e. Local thyroxin administration, however, can terminate this waiting period.

SUMMARY

1. This study was carried out on the frog tadpole in order to determine whether or not the maturation of the center for the corneal reflex, located in the medulla oblongata, is under direct control of thyroid hormone.
2. Normally the reflex appears an average of 4 days prior to the emergence of the forelimbs.
3. Addition of thyroxin to the water in which the tadpoles are kept advances the first appearance of the reflex less than it does other metamorphic changes. The onset of the reflex precedes forelimb emergence by less than 4 days, or even follows forelimb emergence.
4. In contrast, the local application of the hormone in the mid-larval tadpole to the vicinity of the reflex center (implantation in the fourth ventricle of fresh rat thyroid fragments or of agar pellets previously soaked in thyroxin solutions) has the opposite effect. The onset of the reflex is advanced more than are other metamorphic events. In the older group of tadpoles of Series A this advance amounted to 8 days, on the average, with a period of 2 weeks required after implantation to bring the reflex mechanism to a functional state.
5. In those cases in which the implant comes to lie nearer one wall of the medulla than the other, the reflex first appears earlier on that side, presumably because of the greater thyroxin concentration.
6. Some of the hormone from the implants, circulating in the blood stream, accelerates general metamorphosis also; in the case of the strongest thyroxin solution (1:500) the general effect of the implant is so great as to mask the local effects. In such a series (C) the onset of the reflex occurred only 5 days prior to forelimb emergence. In thyroxin-treated controls whose metamorphosis has been accelerated to an equal degree the reflex usually appears at the time of forelimb emergence. The difference of 5 days between the two groups can be ascribed to local effects of the implant.
7. Implants of thyroxin-soaked agar pellets into the fourth ventricle of premetamorphic tadpoles results in the onset of the reflex in from 12 to 36 hours. This indicates that in these older animals the reflex center is essentially ready to function, requiring only the final stimulation provided by the hormone.
8. Similar implants made into young mid-larval tadpoles are ineffective in producing a premature appearance of the reflex, although they do speed up body metamorphosis for a time. Apparently the reflex mechanism in these younger tadpoles has not yet self-differentiated to the point where thyroxin can be effective in setting it into action.

LITERATURE CITED

- ADAMS, A. E., and MERWIN, R. M. 1939. A study of grafts of mouse thyroid glands in newts. *Anat. Rec.*, Suppl., 75:128.
- ALLEE, W. C.; COLLIAS, N. E.; and LUTHERMAN, C. Z. 1939. Modification of the social order in flocks of hens by the injection of testosterone propionate. *Physiol. Zool.*, 12:412.
- BALL, J. 1937. The effect of male hormone on the sex behavior of female rats. *Psychol. Bull.*, 34:725.
- DOMM, L. V. 1929. Testis grafts in bilaterally ovariectomized fowl. *Anat. Rec.*, 44:204.
- . 1937. Observations concerning anterior pituitary-gonadal interrelations in the fowl. Cold Spring Harbor Symp. on Quant. Biol., 5:241.
- GUDERNATSCH, J. F. 1912. Feeding experiments on tadpoles. I. The influence of specific organs given as food on growth and differentiation. *Arch. f. Entw'mech. d. Org.*, 35:457.
- HARTWIG, H. 1940. Metamorphose-Reaktionen auf einen lokalisierten Hormonreiz. *Biol. Zentralbl.*, 60:473.
- HELFF, O. M. 1928. Studies on amphibian metamorphosis. III. The influence of the annular tympanic cartilage on the formation of the tympanic membrane. *Physiol. Zool.*, 1:463.
- IDZKOWSKY, I. 1932. Some reactions of an amphibian host, *Triturus viridescens*, against implanted mammalian tissue. *Anat. Rec.*, Suppl., 54:60.
- KOLLROS, J. J. 1942a. Experimental studies on the development of the corneal reflex in Amphibia. I. The onset of the reflex and its relationship to metamorphosis. *Jour. Exper. Zool.*, 89:37.
- . 1942b. Localized maturation of lid-closure reflex mechanism by thyroid implants into tadpole hindbrain. *Proc. Soc. Exper. Biol. and Med.*, 49:204.
- LERMAN, J. 1941. Glandular physiology and therapy: the physiology of the thyroid gland. *Jour. Amer. Med. Assoc.*, 117:349.
- LITTLE, M. E. 1929. The transplantation of mammalian tissue into amphibian tadpoles. *Proc. Soc. Exper. Biol. and Med.*, 26:372.
- NOBLE, G. K.; KUMPF, K. F.; and BILLINGS, V. N. 1936. The induction of brooding behavior in the jewel fish. *Anat. Rec.*, Suppl. 1, 67:50.
- RIDDLE, O.; BATES, R. W.; and LAHR, E. L. 1935. Prolactin induces broodiness in fowl. *Amer. Jour. Physiol.*, 111:353.
- ROSS, D. A., and SCHWAB, R. S. 1939. The cortical alpha rhythm in thyroid disorders. *Endocrinology*, 25:75.
- STONE, C. P. 1927. The retention of copulatory ability in male rats following castration. *Jour. Comp. Psychol.*, 7:369.
- . 1932. The retention of copulatory ability in male rabbits following castration. *Jour. Gen. Psychol.*, 40:296.
- . 1939. "Sex drive," chap. xxiii in EDGAR ALLEN, C. H. DANFORTH, and E. A. DOISY (eds.), *Sex and internal secretions*. Baltimore: Williams & Wilkins.
- WEISS, P. 1941. Self-differentiation of the basic patterns of co-ordination. *Comp. Psychol. Monog.*, 17, No. 4.
- . 1942. Lid-closure reflex from eyes transplanted to atypical locations in *Triturus torosus*; evidence of a peripheral origin of sensory specificity. *Jour. Comp. Neurol.*, 77:131.

EXPERIMENTAL STUDIES ON THE DEVELOPMENT OF THE CORNEAL REFLEX IN AMPHIBIA

III. THE INFLUENCE OF THE PERIPHERY UPON THE REFLEX CENTER

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TWO FIGURES

The normal course of development of the corneal (lid-closure) reflex and the manner in which it is modified by nutritional and endocrine influences has been described previously (Kollros, '42, '43). It is the purpose of the present paper to consider in what manner the alteration of the normal peripheral nerve connections can modify the expression of this reflex.

In *Triturus* the reflexogenous area for the corneal reflex includes, at metamorphosis, nearly the entire dorsal surface of the head, extending in some cases as far caudad as the base of the gills. After metamorphosis the extent of the reflexogenous area decreases, so that the region of the ear capsule no longer lies within it, or else only its anterior portion does. Tactile stimuli applied to the skin in this region can no longer elicit the corneal reflex. Instead, the head is jerked away from the point of stimulation (Weiss, '42).

In his work on *Triturus*, Weiss transplanted supernumerary eyes in place of the nasal organ or into the ear capsule. These animals were studied 2 or 3 months after metamorphosis, at a time when the reflexogenous area had already been reduced

¹This research was done under the direction of Dr. Paul Weiss in partial fulfillment of the requirements for the degree of Doctor of Philosophy. It has been aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of The University of Chicago.

in extent. Stimuli applied to the cornea of the graft were essentially as effective in producing retraction of the host eye as were those applied to the host eye itself. With but few exceptions, stimuli applied to the skin in the same region on the unoperated side failed to elicit the retraction of the eyeball. If retraction was elicited, very strong stimuli were required. Ususally the head-jerk alone was noted. These experiments prove that:

“The sensory periphery, far from being forced into a rigid response relation with the centers by unfailingly stereotyped and predetermined fiber connections, takes an active part in establishing selective correspondence with the central system” (Weiss, '42, p. 164).

Weiss further extends the hypothesis of progressive modulation to include the corneal and cutaneous innervation. Previously this hypothesis had been advanced for the modulation of motor and proprioceptive neurones by their respective terminal organs (Weiss, '36, '41).

These recent studies of Weiss on the lid-closure reflex did not include work on the development of the reflex. It has not been determined whether the reflexogenous area centering about the grafted eye develops as a separate unit premetamorphically, fusing before metamorphosis with that of the host eye, and later becoming isolated again as a separate entity, or whether it first assumes independence only when the reflexogenous zone shrinks after metamorphosis. If the former alternative were the correct one, it would be immediately evident that the cornea plays an active role in modulating the sensory neurones by which it is innervated. This is the interpretation favored by Weiss. The other alternative noted by him was the possibility that the corneal innervation was of a primitive type, and that postmetamorphically the cutaneous innervation of the head becomes skin-modulated, resulting in encroachment upon and reduction in the extent of the reflexogenous area of the lid-closure reflex. This restriction never becomes complete, for the reflexogenous area always includes

some skin about the eye. It will be seen that the first alternative noted above is the correct one.

The lid-closure reflex is elicitable from a considerable extra-corneal area of the skin as well as from the cornea. Will a reduction of the cornea influence the extent of the reflexogenous area? Is the presence of cornea necessary for the elicitation of the reflex? Will its removal influence the thresholds of the various skin areas from which the reflex is elicitable? Can Weiss' results on *Triturus* be duplicated in forms in which the reflexogenous area fails to shrink post-metamorphically, or shrinks only slightly, as for example, in *Amblystoma* and *Rana*? The answers to these questions are now available.

MATERIALS AND METHODS

The urodeles used in this study were embryos and larvae of *Amblystoma punctatum*, *A. opacum*, *A. tigrinum*, and *Triturus torosus*. The anurans used were embryos and tadpoles of *Rana pipiens*, and tadpoles of *Bufo fowleri*. The young urodeles were fed *Daphnia*, mosquito larvae and pupae, and *Enchytraeus*. Older larvae and adults were fed liver. Anuran tadpoles were fed boiled lettuce. Unless otherwise noted, animals were fed at nearly maximal levels. Tactile stimuli in the earlier experiments were administered with a human hair protruding some 5 to 7 mm. from its mount. The hair was applied with its side rather than its tip. Stimulation was considered "light" if the hair was bent but slightly during application, "medium" if bent from 15° to 30°, and "strong" if bent more than 30°. In the later experiments stimuli and responses were graded according to the method described by Weiss ('42). Four fibers of different stiffness were mounted in glass tubes. Each was applied sideways to the pan of an analytical balance, and the counterweight necessary to bend it about 15° was determined. These values for hairs nos. 1 to 4 were 3, 18, 57, and 300 mg. respectively. Four modes of stimulation were used:

1. Single point contact.
2. Repeated touch to the same spot (temporal summation).
3. Single stroke over a larger area (spatial summation).
4. Repeated strokes (temporal and spatial summation).

The left (L) and right (R) sides of the head were then divided into five areas (fig. 1). Areas 1 to 4 correspond to those of Weiss with the exception that area 1 is somewhat

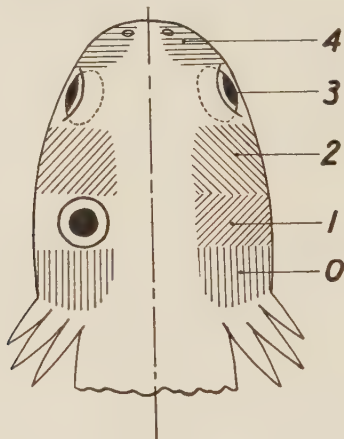


Fig. 1 Map of test areas of stimulation. Supernumerary eye is shown in area 1 on the left (over the ear capsule).

restricted posteriorly by the addition of a new area. Area 0 has been added to include the zone between the ear capsule and the base of the gills.

Four major types of response can be distinguished:

1. Twitch.
2. Momentary partial retraction of the eyeball.
3. Momentary complete retraction of the eyeball.
4. Sustained (1 second or longer) complete retraction of the eyeball.

The type of stimulation and the response elicited may then be recorded in the following order: side stimulated, area stimulated, strength of stimulus, mode of stimulation, and response. Thus, L 3 1 4 : 3 would mean that on the left (L) side the normal corneal area (3) had been stimulated

by the finest fiber (1), using repeated strokes (4), and that the response had been a momentary complete retraction of the bulb (3).

All stimulations and observations were performed under the low power ($20\times$) of a wide field binocular dissecting microscope. Stimulation of larvae was done under water, and of adults in air. The movements of the animals were unrestrained during stimulation. All operations were performed in sterile Holtfreter's or amphibian Ringer's solutions. Chloretone anesthesia was employed in operations on embryos, and ethyl urethane in those on larvae.

All remarks concerning the developmental stages of urodele embryos refer to the stages described by Harrison for *Amblystoma punctatum*. Stages referring to *Rana pipiens* embryos are as described by Shumway ('40).

EXPERIMENTAL

Heterotopic grafting of supernumerary eyeballs

A. Amblystoma. Supernumerary eyeballs were grafted in place of the otocyst or just posterior to it (areas 1 and 0 respectively) in fifteen embryos of *A. opacum* and *A. punctatum* in stages 37 to 45. When the former site was used it was found that the eyeball was larger than the otocyst which had been removed, and the graft site had to be enlarged slightly by clearing away some of the surrounding tissue. Posterior to the otocyst the graft site was prepared by removing the mesenchyme or newly developed muscle between the otocyst and the afferent branchial arteries running to the gills. Owing to the presence of these blood vessels, this graft site could not be incised as deeply as at the otocyst level, and the graft usually protruded somewhat.

The corneae of the grafts remained transparent. They were innervated at an early stage, as was determined by touching them lightly, whereupon the animal either depressed its gills or swam away. In the early work on these animals only the human hair (fiber no. 2) was used as a test stimulus. During

the premetamorphic period the development of the reflex proceeded normally, as described before (Kollros, '42). The reflexogenous area spread at a normal rate, and at no time was the reflex elicitable from the cornea of the graft prior to the normal extension into the graft region of the reflexogenous area of the host eye. The sensitivity of the graft appeared to be identical with that of the skin located symmetrically on the opposite side of the head, rather than like that of the host cornea (light touch elicited a partial retraction of the bulb). When the results of Weiss' studies on *Triturus* were made available, the possibility of finding evidence of both premetamorphic and postmetamorphic modulation was reinvestigated, using the graded series of stimulation fibers.

Eyes from *Amblystoma punctatum* larvae 20 to 25 mm. in length were grafted homoplastically just posterior to the ear capsule (area 0) of six larvae ranging in length from 28 to 40 mm. Only in the largest one of these hosts was the reflex elicitable prior to the operation, and then only from the restricted area of the cornea and the developing lids. The animals were subsequently maintained at submaximal nutritional levels for the following 2 months, until metamorphosis. During this period the corneal reflex developed. In none of the animals was a discrete and separate reflexogenous area found centering around the grafted eye. The reflex was not elicitable from the graft until after the incorporation of the area of the graft into the reflexogenous area of the host eye. There is a possibility, however, that if the animals had been tested daily, or on alternate days, instead of at longer intervals, such a discrete area might have been found (see sect. B on *Triturus*). The evidence for this statement rests on the fact that even at the first elicitation of the reflex from the grafts, the thresholds were somewhat lower than those of the area just anterior to the grafts (see the protocols of cases 1249 and 1268). For the symbols used in the description, see the section on materials and methods.

Case 1249. *Amblystoma punctatum*. Graft at right in area 0. The first test was taken 2 days before reduction of the gill filaments began.

L 0 1 3 : 0	R 0 1 3 : 1
L 0 1 4 : 0	R 0 1 4 : 2
L 0 2 3 : 2	R 0 2 3 : 3
L 1 1 3 : 0	R 1 1 3 : 0
L 1 2 3 : 2	R 1 2 3 : 2

Second test, 3 weeks after the first. Postmetamorphic feeding has begun.

L 0 1 3 : 0	R 0 1 3 : 2
L 0 1 4 : 0	R 0 1 4 : 3
L 0 3 3 : 0	R 0 3 3 : 4
L 1 3 3 : 0	R 1 3 3 : 3
L 2 2 3 : 2	R 2 2 3 : 2
L 2 3 3 : 3	R 2 3 3 : 3

Third test, 6 weeks after the first.

L 0 1 1 : 0	R 0 1 1 : 0
L 0 1 3 : 0	R 0 1 3 : 2
L 0 1 4 : 0	R 0 1 4 : 3
L 2 1 3 : 0	R 2 1 3 : 2
L 2 1 4 : 1	R 2 1 4 : 3
L 2 2 3 : 1	R 2 2 3 : 3

Case 1268. *Amblystoma punctatum*. Graft at right in area 1. Test taken at the time of gill reduction (metamorphic climax).

L 1 1 1 : 0	R 1 1 1 : 4
L 1 1 3 : 0	R 1 1 3 : 4
L 2 1 3 : 0	R 2 1 3 : 0
L 2 1 4 : 2	R 2 1 4 : 2
L 3 1 1 : 4	R 3 1 1 : 4

As can be seen from the protocols, the postmetamorphic thresholds for the cornea of the graft remain the same as at metamorphosis, or become even lower, while the thresholds for the skin opposite the graft increase until only the strongest stimuli elicit the reflex. There is, however, seldom a complete loss of the capacity for eliciting the reflex from any skin area that once showed such a capacity in the larva.

B. Triturus. Eyes of *Amblystoma punctatum* larvae about 20 mm. in length were grafted dorsal to the ear capsule or just posterior to it in ten *Triturus torosus* larvae about 25 mm. in length. These animals were fed at levels well below maximal. Prior to metamorphosis they were tested at intervals of a few days, and less frequently for several months following metamorphosis. In two larvae the region of the graft developed as an independent reflexogenous area, the

reflex being elicited from it at a time when no stimulus applied to the area immediately anterior to it would elicit the reflex. The thresholds of the graft were at first much higher than those of the host cornea (see protocol of case 307).

Case 307. *Triturus torosus* larva with an extra eye grafted just posterior to the right ear capsule (area 0). Tests made 5 days after the onset of the reflex.

L 0 4 4 : 0	R 0 4 4 : 1 or 2
L 1 4 4 : 0	R 1 4 4 : 0
L 2 4 4 : 1	R 2 4 4 : 1
L 3 2 3 : 2	R 3 2 3 : 2
L 3 2 4 : 4	R 3 2 4 : 4
L 4 2 4 : 3	R 4 2 4 : 3
L 4 2 3 : 1	R 4 2 3 : 1

As the reflexogenous area expanded, it spread over the cornea of the graft, and at all head levels the thresholds on the two sides were symmetrical. Shortly before metamorphosis, or just at the start of gill reduction, the threshold of the graft was again somewhat lower than that of the identical area on the opposite side, although at that phase not necessarily lower than that of area 1 anterior to it. See the group of protocols for cases 307, 308, and 310.

Records at metamorphic climax of three *Triturus torosus* with extra eyes grafted just posterior to the ear capsule.

Case 307. Graft at right.

L 0 1 4 : 0	R 0 1 4 : 3
L 0 1 1 : 0	R 0 1 1 : 2
L 1 1 4 : 2	R 1 1 4 : 3
L 1 1 3 : 1	R 1 1 3 : 3
L 1 1 1 : 0	R 1 1 1 : 3
L 3 1 1 : 3 or 4	R 3 1 1 : 3 or 4

Case 308. Graft at left.

L 0 1 1 : 0	R 0 1 1 : 0
L 0 1 3 : 1	R 0 1 3 : 0
L 0 1 4 : 2 or 3	R 0 1 4 : 0
L 1 1 3 : 2	R 1 1 3 : 2

Case 310. Graft at left.

L 0 2 3 : 2 or 3	R 0 2 3 : 0
L 0 1 4 : 1	R 0 1 4 : 0
L 1 2 3 : 3	R 1 2 3 : 3
L 4 1 1 : 4	R 4 1 1 : 3

These results indicate that the thresholds of the cornea of the graft have gradually decreased. The area immediately about the graft is more sensitive than the corresponding area on the opposite side, but most of areas 3 and 4 are equally sensitive on the two sides. Subsequently the cornea of the graft becomes increasingly sensitive, and at the same time the threshold differences of areas 2 and 3 on the two sides become marked. This has already been shown by Weiss ('42). These progressive changes in the thresholds are illustrated in figure 2. The eight remaining animals had the same post-metamorphic history, but before metamorphosis they failed to display a separate reflexogenous area about the grafted eyeball.

In the present study no experiments with supernumerary eyes have been performed with *A. tigrinum*. However, in older larvae of *A. tigrinum* the supernumerary eyes display a much lower threshold than does the interocular area immediately anterior to them.²

It should be noted that the elicitation of the reflex from the graft in *Triturus* at a time when the reflexogenous area centered about the host eye had not yet extended to the graft level indicates an active specialization, or modulation, of the nerve fibers by the graft. These results corroborate those of Weiss on metamorphosed *Triturus* and on larval *A. tigrinum*. The process continues after metamorphosis until the thresholds at the host and graft eyes are equal, or nearly so.

C. Rana. Eyeballs from *Rana pipiens* tadpoles about 35 mm. in length were transplanted into the ear capsules (or just posterior to them) of twelve *R. pipiens* tadpoles about 40 mm.

² Personal communication by Dr. Paul Weiss.

in length. Two additional grafts were made in which the eyeballs replaced the nasal organ. In eight animals of the former group the skin which covered the grafts never became completely transparent, and at metamorphosis it was converted into normal head skin. At metamorphic climax, when the reflex was elicitable from the host cornea, stimulation of

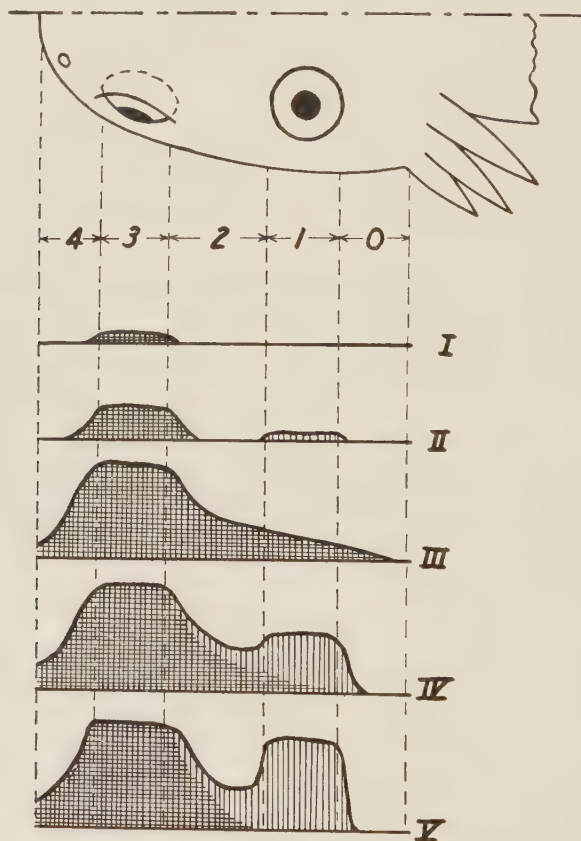


Fig. 2 The profiles illustrate diagrammatically the thresholds for the corneal reflex in *Triturus torosus* during each of five different periods. Ordinates indicate sensitivity (reciprocal threshold values); the higher the curve, the lower the threshold. Areas indicated by vertical hatching represent the profile of thresholds on the side with the graft; horizontal hatching represents the profile of thresholds on the opposite side. I, at the first appearance of the reflex. II, 5 days later. III, shortly before the start of gill reduction. IV, at metamorphic climax. V, 1 to 2 months after metamorphosis.

the area of the graft was ineffective in eliciting the reflex except occasionally and upon the use of very strong stimuli. In the four remaining animals of the group, the cornea which formed over the eyeball became completely transparent. It was maintained in this condition during and after metamorphosis. The area of the graft bordered on the posterior edge of the normal reflexogenous area. The stimulus usually required to elicit the reflex from the skin of this area is very strong. In these four animals, however, such was not the case, for the strength of the stimulus needed to elicit the retraction of the host eyeball from the cornea of the graft was nearly as weak as that required when the host cornea itself was stimulated. See the protocol for case 1224.

Case 1224. *Rana pipiens* with extra eyes grafted into right area 1.

Tested 2 days after the appearance of the forelegs.

L 1 4 3 : 0 R 1 4 3 : 2

Tested 10 days after the appearance of the forelegs.

L 1 2 2 : 0 R 1 2 2 : 2
L 1 2 3 : 0 R 1 2 3 : 3

Tested 13 days after the appearance of the forelegs.

L 1 2 1 : 0 R 1 2 1 : 3
L 1 2 3 : 0 R 1 2 3 : 3
L 3 2 1 : 3 R 3 2 1 : 3

Unlike the condition in *Triturus*, there was no area between the graft and the host eyeball from which the reflex could not normally be obtained. The two successful grafts to the nasal area also displayed an increased sensitivity as compared to the unoperated side opposite them (see protocol for case 1233).

Case 1233. *Rana pipiens* with extra eye grafted in place of the right nasal organ. First test, 7 days after the appearance of the forelegs. The second test, 5 days later, gave identical results.

L 4 2 1 : 0 R 4 2 1 : 3
L 4 2 3 : 0 R 4 2 3 : 3

Third test, 19 days after the appearance of the forelegs.

L 4 2 1 : 0 or 1 R 4 2 1 : 3
L 4 2 2 : 3 R 4 2 2 : 4
L 3 2 1 : 3 R 3 2 1 : 3

Reduction in the size of the cornea

What would be the effect of eliminating the cornea, and of replacing it by the surrounding skin? Would the corneal reflex appear? If so, would the reflexogenous area be of the same extent as in normal animals? Would the thresholds be altered?

It is known that without an eyeball or lens underlying it, presumptive corneal epidermis is incapable of differentiating into cornea; interposition of mesenchyme between the eyeball and epidermis is equally effective in preventing corneal differentiation; the size of the cornea depends upon the size of the eyeball and upon the extent of the area of contact between the eyeball and epidermis; an eyeball is necessary for the normal maintenance of a cornea, and enucleation leads to a clouding of the cornea and eventually to its complete obliteration (see Mangold, '31 for a review of work on corneal development and maintenance). This dependence of the cornea upon the eyeball and lens permits a variety of methods to be used in eliminating the cornea proper.

I. Reduction of the eye rudiment

A. Urodeles. A portion of the right eye vesicle was removed from each of thirty-seven *Amblystoma tigrinum*, *A. punctatum*, and *Triturus torosus* embryos in stages 35 to 44. The eyeballs and corneae which developed could be expected to be smaller than normal, the size varying inversely with the proportion of the rudiment which had been removed. In one animal both the eyeball and cornea failed to develop. In another animal a diminutive eyeball and cornea were formed. The eye was immobile, and no reflex could be detected. In a third case the eyeball was fully formed, but separated from the epidermis by mesenchyme. No cornea developed. As the eyeball was immobile, presumably due to adhesions, no reflex was detected. In thirty cases, however, the eyeballs, and consequently the corneae, were present but smaller than normal. The corneal reflex was elicitable from all of the corneae. In twenty-

six of these animals the thresholds for the reflex developed symmetrically on operated and unoperated sides. In the four others the thresholds appeared asymmetrical, with a larger reflexogenous area on one side than on the other. In two of these the area was greater on the unoperated side, while in the two others it was the operated side which was favored.

In the four remaining animals mesenchyme had become interposed between the eyeball and the epidermis. In three of them the cornea failed entirely to develop, while in one it appeared only as a small, translucent slit. The reflexogenous areas were approximately symmetrical on the two sides. The thresholds of the skin over the orbit on the operated side appeared to be slightly lower than those of the normal cornea. This probably is in part due to the mechanical arrangements of the moving parts, and not completely due to a lowering of the sensitivity of the skin replacing the cornea. Usually the eyeball is rather firmly fused to the overlying cornea; consequently, in the period preceding the formation of the conjunctival sac and eyelids, retraction of the bulb deforms the skin or cornea at the edge of the orbit. The eyeballs of the four animals lacking a cornea were not fused to the overlying skin; hence, at a given premetamorphic stage, light stimuli were adequate to elicit a slight withdrawal of the bulb on the operated side, whereas on the opposite side the stiffness of the attached cornea prevented eye retraction. Medium or strong stimuli did result in the retraction of the bulb on the unoperated side. No postmetamorphic study of these animals was made. Had it been made, a change in the sensitivity of the reflexogenous areas on the operated and unoperated sides might have been noted, favoring the unoperated side. This change from the larval condition should be expected as a result of the formation of the conjunctival sac on the unoperated side.

B. Anuráns. Portions of the left eye vesicle were removed from two *Rana pipiens* embryos at stage 18. In one of them the lens rudiment had also been removed and remained absent.

The eyeballs and corneae of the operated sides were reduced to about one-third of the normal diameter. As in urodeles, the reflex developed at the normal time on both sides, and the reflexogenous areas spread at the same rate and to the same extent.

Apparently, in urodeles and anurans alike, the size of the cornea has nothing to do with the extent of the normal reflexogenous area, nor does the complete absence of cornea (*Amblystoma*) prevent the development of the reflex.

II. Mediolateral reversal of the eyeball

In order to eliminate the cornea completely, while still retaining a movable structure in the orbit to which the retractor bulbi muscle might attach, it was decided to reverse the eyeball upon its mediolateral axis. The lens would then face medially, and the cut stump of the optic nerve would face laterally toward the skin. Since the configurations of the medial and lateral surfaces of the larval eyeball differ, it was found that the eyeball fitted rather poorly into its original orbit, generally bulging against the cornea, which had been left intact. To obviate this difficulty, donors were selected somewhat smaller than hosts.

A. Urodeles. Eye vesicles were transplanted homoplastically from fourteen *A. opacum*, *A. punctatum*, *A. tigrinum*, and *Triturus torosus* embryos in stages 35 to 42, into the orbits of slightly older embryos, reversing the mediolateral axis. Later, in four of these animals (three lacking a cornea, and a fourth with a diminutive one), the eyes were found to be immobile. No reflex could be expected. Of the remaining ten, only one had a cornea. All ten developed the reflex simultaneously on operated and unoperated sides. The thresholds were approximately the same on the two sides, except for the cases in which the eyeball remained free in the orbit, unattached to the overlying skin. In these animals the thresholds in the orbital region appeared to be slightly lower on

the operated side than on the unoperated one. This can be explained just as in the similar cases of the preceding section.

Reversals of the mediolateral axis of the eyeball were also performed in eight larval *A. punctatum* and *A. opacum* ranging in length from 20 to 45 mm. The largest animal already displayed the reflex at the time of operation. In performing this operation an incision was made dorsal to the eyelid and through to the orbit. The eye muscles and the optic nerve were cut, and the eyeball lifted out through the incision. Thereby the cornea is divided into its two constituent layers, the inner one remaining with the eyeball, and the outer one covering the orbit as before. This is reminiscent of the double condition of the cornea in the anuran tadpole (Harms, '23). Finally an eyeball from a donor slightly smaller in size was inserted into the orbit.

At metamorphosis five of these animals lacked a cornea, while the other three had small, slit-like translucent areas still present. These last three, and two of those lacking a cornea, developed tiny eyelids. In one animal the operated eye remained immobile. The remaining seven developed the reflex, with the reflexogenous areas and the thresholds throughout being nearly the same on the two sides. These results indicate, first of all, that the complete absence of the cornea does not prevent normal reflex development, and secondly, that the retractor bulbi muscle had inserted upon the original external surface of the bulb. The last result is somewhat similar to the finding of Hall ('41), that abnormally placed ear capsules displayed abnormal points of attachment of the depressor mandibulae muscle. In the present operations the extrinsic eye muscles inserted upon the reversed bulb in from 12 to 48 hours after the operation, for the movement of the eyeball within the orbit could be seen in that interval. One of the animals displaying the reflex in the absence of the cornea lacked the eyeball also, because after replantation the eyeball had been resorbed. The retractor bulbi muscle in this instance inserted upon the skin covering the orbit, and stimulation of the skin brought about its retraction.

B. Anurans. One eye vesicle in each of three *Rana pipiens* embryos was reversed on its mediolateral axis in stage 18. Two larval animals of 40 mm. were similarly treated. In both of the operated larvae the eyeballs were subsequently partially resorbed, being reduced to about two-fifths of their normal diameter. In both specimens the cornea clouded slowly until metamorphosis, and at that time in one of them it became converted into normally pigmented head skin. All five of the animals displayed the reflex on both sides, although only one of the group had a cornea. Thresholds were equal on the two sides.

These experiments show again that the corneal reflex does not require the presence of normal cornea for its appearance, its spread, or its maintenance. In fact, in one of the *A. punctatum* specimens the reflex was obtained when both the cornea and eyeball were absent.

III. Substitution of the ear capsule for the eyeball

In order to eliminate both the cornea and the eyeball as factors in the development of the reflex, eye vesicles of embryos were replaced by otocysts, or eyeballs of larvae by ear capsules (labyrinths) of other larvae.

A. Urodeles. The otocyst was substituted for the eye vesicle in five *A. punctatum* embryos in stages 38 to 43. In four of them the cornea failed to develop, while in one it was present as a translucent area during the larval period, but was transformed into head skin at metamorphosis. In this last specimen, and in one other, abortive attempts at lid formation were noted. The reflex developed normally in four of the cases, except for somewhat higher thresholds in the orbital region on the operated side. The presence of the reflex indicates that the retractor bulbi (and the other extrinsic eye muscles as well) had inserted upon the ear capsule. This contrasts with the observations of Hall ('41) that the depressor mandibulae muscle failed to attach to the eyeball or lens when these were transplanted in place of the ear capsule.

In seven *A. punctatum* and *A. opacum* larvae the eyeball was enucleated and replaced by an ear capsule. Within twelve hours, usually, the eye muscles had inserted upon the ear capsule, and in the oldest animals retraction and other eye movements could be elicited. At metamorphosis the clouded corneae of five of the animals were converted into normally pigmented head skin, while in the other two the corneae remained small and transparent. The reflex developed normally on both sides in all seven animals. Thresholds were higher in the orbital area on the operated side, but elsewhere they were approximately normal.

B. Anurans. The ear capsule was grafted in the place of the eyeball in eight *Rana pipiens* tadpoles of various sizes. As metamorphosis approached, the cornea became increasingly cloudy. In three of the animals the corneae were converted into normal skin at metamorphosis, while in the other five the corneae persisted as small, translucent slits. The edges of these slits (lids) showed the lowest threshold for the reflex. Except for the converted corneal area itself, in which the thresholds were higher than on the unoperated side, the threshold values for the reflex were distributed symmetrically.

It is therefore apparent that amphibians require neither an eyeball nor a cornea in order to develop the corneal reflex. It must be noted that in all cases in which the cornea was missing, the corneal tissue had either been transformed into skin or replaced by surrounding skin. In either case, the skin covering the orbit developed from the skin within the normal reflexogenous area. This raises the question of whether the reflex would likewise be displayed if skin from outside the reflexogenous area were to replace the cornea. Experiments dealing with this problem have been started, but the results are not yet available.

Continuous regeneration of the cornea

One series of experiments was designed to determine whether the modulation of the sensory nerve fibers of the

corneal zone occurred concurrently with the maturation of the central mechanism of the reflex, or whether these two independent processes took place at different times. The experiment consisted of the removal of the outer cornea of the tadpole at 5-day intervals until a few days before the start of metamorphic climax was expected. These operations forced the cornea to remain in a regenerating (thus relatively undifferentiated) condition until shortly before the onset of the reflex. The question was whether such treatment would delay the onset of the reflex until an advanced stage in corneal differentiation had been reached. The answer was in the negative. The operations were carried out on seven *Rana pipiens* and two *Bufo fowleri* tadpoles. In seven other *Rana* tadpoles the skin of nearly the entire reflexogenous area was removed repeatedly. In some cases as many as ten operations were performed on one tadpole, and the average number of such operations was six. The final operation in all cases was performed about 5 to 7 days before the onset of the reflex was expected. In all cases the reflex appeared at the expected time, on both operated and unoperated sides. On the operated side the reflex was elicitable at first only from the periphery of the regenerated area; but within 10 to 14 days after first appearing, the reflex could be elicited from any portion of the regenerated cornea. This delay is attributable to the time required for new nerve fibers to grow in and establish functional connections.

DISCUSSION

The experiments with grafts of supernumerary eyes in the otic and postotic areas demonstrate that the cornea of the *Triturus torosus* larva, and perhaps that of *Amblystoma punctatum* as well, must be regarded as actively influencing the relationship of the sensory periphery to the central mechanism of the reflex. In *Triturus* this influence of the cornea of the graft is demonstrable in two periods. For a short time, just after the first appearance of the reflex, a

stimulus to the graft elicits the reflex, while a stimulus to the skin around the graft and in front of it fails to do so. For the remainder of the premetamorphic period, the influence of the graft is not demonstrable. After metamorphosis the sensitivity of the supernumerary cornea increases for about 2 months, finally reaching approximately the same level as that of the cornea of the host eye (Weiss, '42). In *Amblystoma* the postmetamorphic phase is similar to that of *Triturus*, but the brief premetamorphic period was not observed in *A. punctatum*. This was probably due to the long interval between observations. Had the observations been less distantly spaced, a separate reflexogenous area about the graft might have been noted premetamorphically. That it might have appeared is suggested by the fact that when the reflex was first elicitable from the graft, the sensitivity at the graft was higher than for the region immediately anterior to it, although later in the premetamorphic period this difference in thresholds disappeared. In *A. tigrinum*, in contrast, the supernumerary eyes display a much lower threshold than does the interocular area immediately anterior to them. This condition persists to metamorphosis.

In general, therefore, *Amblystoma* and *Triturus* can be considered as demonstrating the modulating influence of the cornea upon its sensory nerve supply to an approximately equal extent.

Can the conclusions which have been drawn from the results of the experiments with supernumerary eyes be applied to the normal cornea? Does an increase in sensitivity and in the extent of the reflexogenous area incident to the addition of a cornea to the head imply that the loss of a cornea will result in the lowering of sensitivity and in the shrinkage of the reflexogenous area? This last question can be answered in the negative. No matter how small the cornea may be, how opaque, in fact, even in its complete absence, the reflex in both *Triturus* and *Amblystoma* can still be elicited from the normal reflexogenous area; and except over the orbit itself, the sensitivity of

the skin will not be lowered. If the size of the cornea is reduced by removing a portion of the eye vesicle, the resulting discrepancy in the size of the corneae on the two sides persists through metamorphosis. In some instances, following such an operation, so little of the eye rudiment remains that mesenchyme becomes interposed between the diminutive eyeball and the skin, and the cornea fails to form. If the eyeball remains freely movable under the skin, the reflex can still be registered as a depression in the skin over the orbit. After metamorphosis the sensitivity of the skin over the orbit is at about the same level as that of the skin immediately surrounding the orbit, rather than like that of a normal cornea.

Other methods of obliterating the cornea in a larger proportion of cases were devised, while at the same time a movable object to indicate the retractor response was retained in the orbit. The eyeball was reversed on its mediolateral axis, for example, or else it was replaced by an ear capsule (otocyst in the embryo). Results identical with those just described for the cases lacking a cornea were obtained. Even in the complete absence of a cornea throughout the larval and adult stages, the reflex was elicitable from the entire extent of the reflexogenous area, and except for the orbital area, the sensitivity of the skin was identical on operated and unoperated sides.

Whether the appearance of the reflex in the absence of a cornea is due to the presence of "corneal" specificity within the circumorbital skin which renders the latter capable of impressing "corneal" modulation upon its sensory nerve supply, or whether it is due to some other factors, can be decided only by appropriate experiments. Work on this problem has already begun.

CONCLUSIONS

1. In *Amblystoma* and *Triturus* the presence of a supernumerary eye in the otic area on the head modifies the type of response elicitable from that area. Normally stimulation

of the skin there elicits a head flexure (*Triturus*), but stimulation of the extra cornea results in a retraction of the host eye on the same side (Weiss, '42).

2. In *Triturus*, *Amblystoma*, and *Rana pipiens* with similar grafts there is a rise in the sensitivity of the skin lying between host and graft eyes to tactile stimuli, the reflex being elicitable with much weaker stimuli than normally.

3. In *Triturus*, and probably in *Amblystoma*, too, this influence of the graft is exerted during the larval period when the reflexogenous area is expanding. The action continues throughout and beyond metamorphosis. In *Triturus*, in which the reflexogenous area usually shrinks after metamorphosis, the extra eye not only prevents shrinkage of the area and the corresponding decrease in sensitivity, but actually raises the sensitivity, at least locally. In *Amblystoma* and *Rana*, likewise, the sensitivity of the graft increases postmetamorphically.

4. Reduction in extent of the host cornea fails to reduce the extent of the reflexogenous area. The sensitivity remains identical on operated and unoperated sides except for the area of the replaced cornea itself, where the sensitivity is somewhat lower.

5. The cornea need not be completely differentiated to permit modulation of the sensory nerve fibers entering it.

LITERATURE CITED

- HALL, E. K. 1941 Experimental modifications of muscle attachments in *Amblystoma*. *Anat. Rec.*, vol. 79, Suppl. no. 2, pp. 29-30.
- HARMS, W. 1923 Brillen bei Amphibienlarven. *Zool. Anz.*, Bd. 56, S. 136-142.
- KOLLROS, J. J. 1942 Experimental studies on the development of the corneal reflex in Amphibia. I. The onset of the reflex and its relationship to metamorphosis. *J. Exp. Zool.*, vol. 89, pp. 37-67.
- 1943 Experimental studies on the development of the corneal reflex in Amphibia. II. Localized maturation of the reflex mechanism effected by thyroxin-agar implants into the hindbrain. *Physiol. Zool.*, vol. 16. In Press.
- MANGOLD, O. 1931 Das Determinationsproblem. III. Das Wirbeltierauge in der Entwicklung und Regeneration. *Ergeb. d. Biol.*, Bd. 7, S. 193-403.
- SHUMWAY, W. 1940 Stages in the normal development of *Rana pipiens*. I. External form. *Anat. Rec.*, vol. 78, pp. 139-147.

- WEISS, P. 1936 Selectivity controlling the central-peripheral relations in the nervous system. *Biol. Rev.*, vol. 11, pp. 494-531.
- 1941 Self-differentiation of the basic patterns of coordination. *Comp. Psych. Monog.*, vol. 17, no. 4.
- 1942 Lid-closure reflex from eyes transplanted to atypical locations in *Triturus torosus*: Evidence of a peripheral origin of sensory specificity. *J. Comp. Neur.*, vol. 77, pp. 131-169.

